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(54) Title: PURIFIED POLYPORUS LACCASES AND NUCLEIC ACIDS ENCODING SAME

(57) Abstract

The present invention relates to isolated nucleic acid constructs containing a sequence encoding a *Polyporus* laccase, and the laccase proteins encoded thereby.

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PURIFIED *POLYPORUS* LACCASES AND NUCLEIC ACIDS
ENCODING SAME

5

Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the 10 purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a basidiomycete, *Polyporus*.

15 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper-containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable 20 phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and 25 humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, *Polyporus* and perfect forms of *Rhizoctonia*. 30 Laccases exhibit a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial

applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

5 Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for
10 several of these have been cloned. For example, Choi et al. (Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima et al. (J. Biol. Chem. 15 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801, 1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the
20 *Neurospora crassa* laccase gene. Saloheimo et al. (J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.

Attempts to express laccase genes in heterologous
25 fungal systems frequently give very low yields (Kojima et al., *supra*; Saloheimo et al., Bio/Technol. 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per liter of active enzyme in lab-scale fermentation (Saloheimo, 30 1991, *supra*). Although laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. Previous attempts to express basidiomycete laccases in recombinant hosts have resulted in very low yields. The

present invention now provides novel basidiomycete laccases which are well expressed in *Aspergillus*.

Summary of the Invention

5 The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Polyporus* laccase. The invention also relates to an isolated laccase encoded by the nucleic acid sequence. Preferably, the laccase is substantially pure. By "substantially pure" is
10 meant a laccase which is essentially (i.e., $\geq 90\%$) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells comprising the claimed nucleic acid sequence, which vectors
15 and host cells are useful in recombinant production of the laccase. The sequence is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus
20 *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the construct of the invention, or progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from
25 the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and
30 phenol resin production.

Brief Description of the Figures

Figure 1 shows the DNA sequence and translation of genomic clone 21GEN, containing LCC1 (SEQ ID NO. 1)

Figure 2 shows the DNA sequence and translation of genomic clone 23GEN, containing LCC2 (SEQ ID NO. 3)

Figure 3 shows the DNA sequence and translation of genomic clone 24GEN, containing LCC3 (SEQ ID NO. 5)

5 Figure 4 shows the DNA sequence and translation of genomic clone 31GEN, containing LCC4 (SEQ ID NO. 7)

Figure 5 shows the DNA sequence and translation of genomic clone 41GEN, containing LCC5 (SEQ ID NO. 9)

Figure 6 shows the structure of vector pMWR1

10 Figure 7 shows the structure of vector pDSY1

Figure 8 shows the structure of vector pDSY10

Figure 9 shows the pH profile of the laccase produced by pDSY2; (A) syringaldazine oxidation; (B) ABTS oxidation.

15 Figure 10 illustrates a comparison of the use of laccase vs. H_2O_2 , with various dye precursors, in hair dyeing, as a measurement of DL^* .

Figure 11 illustrates a comparison of the use of laccase vs. H_2O_2 , with various dye precursors, in hair dyeing, as a measurement of Da^* .

20 Figure 12 illustrates a comparison of the use of laccase vs. H_2O_2 , with various dye precursors and modifiers, in hair dyeing, as a measurement of DL^* .

Figure 13 illustrates a comparison of the wash stability of hair dyed with laccase vs. H_2O_2 .

25 Figure 14 illustrates the light fastness of hair dyed with laccase vs. H_2O_2 .

Detailed Description of the Invention

Polyporus pinsitus is a basidiomycete, also referred to as *Trametes villosa*. *Polyporus* species have previously been 30 identified as laccase producers (Fahraeus and Lindeberg, *Physiol. Plant.* 6: 150-158, 1953). However, there has been no previous description of a purified laccase from *Polyporus pinsitus*. It has now been determined that *Polyporus*

pinsitus produces at least two different laccases, and the genes encoding these laccases can be used to produce relatively large yields of the enzyme in convenient host systems such as *Aspergillus*. In addition, three other genes which appear to code for laccases have also been isolated.

Initial screenings of a variety of fungal strains indicate that *Polyporus pinisitus* is a laccase producer. The production of laccase by *P. pinsitus* is induced by 2,5-xylidine. Attempts are first initiated to isolate the laccase from the supernatant of the induced strains. Anion exchange chromatography identifies an approximately 65 kD (on SDS-PAGE) protein which exhibits laccase activity. The enzyme is purified sufficiently to provide several internal peptide sequences, as well as an N-terminal sequence. The initial sequence information indicates the laccase has significant homology to that of *Coriolus hirsutus*, as well as to an unidentified basidiomycete laccase (Coll et al., *Appl. Environ. Microbiol.* **59**: 4129-4135, 1993). Based on the sequence information, PCR primers are designed and PCR carried out on cDNA isolated from *P. pinsitus*. A band of the expected size is obtained by PCR, and the isolated fragment linked to a cellulase signal sequence is shown to express an active laccase in *A. oryzae*, but at low levels. One of the PCR fragments is also used as a probe in screening a *P. pinsitus* cDNA library. In this manner, more than 100 positive clones are identified. The positive clones are characterized and the ends of the longest clones sequenced; none of the clones are found to be full-length.

Further attempts to isolate a full length clone are made. A 5-6 kb BamHI size-selected *P. pinsitus* genomic library is probed with the most complete cDNA fragment isolated as described above. Initial screening identifies one clone 24GEN(LCC3) having homology to the cDNA, but which is not the cDNA-encoded laccase and also not full length.

Subsequent screening of a 7-8kb BamHI/EcoRI size-selected library indicates the presence of at least two laccases; partial sequencing shows that one, called 21GEN(LCC1), is identical to the original partial cDNA clone isolated, and

5 the second, called 31GEN(LCC4) is a new, previously unidentified laccase. Secondary screenings of an EMBL4 genomic bank with LCC1 as probe identifies a class of clone containing the entire LCC1 insert as well as the 5' and 3' flanking regions. Screening of the EMBL bank with LCC3

10 identifies two additional clones encoding laccases which had not previously been identified, 41GEN(LCC5) and 23GEN(LCC2) and which differed structurally from the other three clones LCC1, LCC3, and LCC4. The nucleic acid and predicted amino acid sequences of each of the laccases is presented in

15 Figures 1-5, and in SEQ ID NOS. 1-10. A comparison of the structural organization of each of the laccases is presented in Table 2. The laccases are generally optimally active at acid pH, between about 4-5.5.

LCC1 is used to create expression vectors, which are in

20 turn used to transform various species of *Aspergillus*. Transformation is successful in all species tested, although expression levels are highest in *Aspergillus niger*. Shake flask cultures are capable of producing 15 or more mg/liter of laccase, and in lab-scale fermentors, yields of over

25 300mg/liter are observed. This is a significant improvement over laccase levels observed previously with other laccases and other fungal host cells.

According to the invention, a *Polyporus* gene encoding a laccase can be obtained by methods described above, or any

30 alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication

of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences

5 encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For

10 expression under the direction of control sequences, a laccase gene to be used according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription

15 of the laccase gene, include but are not limited to the prokaryotic β -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in

20 "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be

25 subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is

30 independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host

cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the laccase DNA sequence should be operably connected to a suitable promoter sequence. The promoter 5 may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, 10 especially in a bacterial host, are the promoter of the lac operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the 15 promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes. In a yeast host, a useful promoter is the *eno-1* promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, 20 *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred 25 are the TAKA-amylase and *glaA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to 30 the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to

replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

5 The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline
10 resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD*, *SC*, *trpC* and *hygB*, a marker giving rise to hygromycin resistance. Preferred for use in an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is
15 the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

It is generally preferred that the expression gives
20 rise to a product which is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different
25 preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase
30 or proteinase gene from *Rhizomucor miehei*, the gene for the α -factor from *Saccharomyces cerevisiae* or the calf preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the signal sequence for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the *Rhizomucor miehei*

aspartic proteinase signal, the *Rhizomucor miehei* lipase signal, the maltogenic amylase from *Bacillus* NCIB 11837, *B. stearothermophilus* α -amylase, or *B. licheniformis* subtilisin. .

5 The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance,
10 Sambrook et al. *Molecular Cloning*, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the
15 recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more
20 likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in
25 connection with the different types of host cells.

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus*
30 *licheniformis*, *Bacillus latus*, *Bacillus brevis*, *Bacillus* *stearothermophilus*, *Bacillus alkalophilus*, *Bacillus* *amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus* *lautus*, *Bacillus megaterium*, *Bacillus* *thuringiensis*, or *Streptomyces lividans* or *Streptomyces*

murinus, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

- 5 The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of
- 10 *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may be selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell.
- 15 Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of
- 20 transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

In a preferred embodiment, the recombinant production of laccase in culture is achieved in the presence of an excess amount of copper. Although trace metals added to the culture medium typically contain a small amount of copper, 5 experiments conducted in connection with the present invention show that addition of a copper supplement to the medium can increase the yield of active enzyme many-fold. Preferably, the copper is added to the medium in soluble form, preferably in the form of a soluble copper salt, such 10 as copper chloride, copper sulfate, or copper acetate. The final concentration of copper in the medium should be in the range of from 0.2-2mM, and preferably in the range of from 0.05-0.5mM. This method can be used in enhancing the yield of any recombinantly produced fungal laccase, as well as 15 other copper-containing enzymes, in particular oxidoreductases.

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating 20 the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. 25 Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as 30 *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA α -amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and

used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474, 1984).

5 It is of particular note that the yields of *Polyporus* laccase in the present invention, using *Aspergillus* as host cell are unexpectedly and considerably higher than has previously been reported for expression of other laccases in other host cells. It is expected that the use of

10 *Aspergillus* as a host cell in production of laccases from other basidiomycetes, such as *Coriolus* or *Trametes*, will also produce larger quantities of the enzyme than have been previously obtainable. The present invention therefore also encompasses the production of such *Polyporus-like* laccases

15 in *Aspergillus* recombinant host cells.

Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figures 1-5. It will also be apparent that the invention 20 encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figure 1-5, but which differ from the specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. Also, reference to Figures 1-5 in the specification and the claims 25 will be understood to encompass both the genomic sequence depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be 30 understood to mean a DNA molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

In addition, the invention also encompasses other *Polyporus* laccases, including alternate forms of laccase which may be found in *Polyporus pinsitus* and as well as laccases which may be found in other fungi falling within 5 the definition of *Polyporus* as defined by Fries, or synonyms thereof as stated in Long et al., 1994, ATCC Names of Industrial Fungi, ATCC, Rockville, Maryland. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by 10 utilization of the methodology described in the present examples, with publicly available *Polyporus* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in isolating laccase genes by standard PCR or southern hybridization techniques. 15 Other named *Polyporus* species include, but are not limited to, *P. zonatus*, *P. alveolaris*, *P. arcularius*, *P. australiensis*, *P. badius*, *P. biformis*, *P. brumalis*, *P. ciliatus*, *P. colensoi*, *P. eucalyptorum*, *P. meridionalis*, *P. varius*, *P. palustris*, *P. rhizophilus*, *P. rugulosus*, *P. 20 squamosus*, *P. tuberaster*, and *P. tumulosus*. Also encompassed are laccases which are synonyms, e.g., anamorphs or perfect states of species or strains of the genus *Polyporus*. Strains of *Polyporus* are readily accessible to the public in a number of culture collections, such as the 25 American Type Culture Collection (ATCC), e.g., ATCC 26721, 9385, 11088, 22084, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), e.g., DSM 1021, 1023, and 1182; and Centraalbureau Voor Schimmelcultures (CBS), e.g., CBS 678.70, 166.29, 101.15, 276.31, 307.39, 334.49, and 332.49. 30 The invention also encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80% homology, preferably at least about 85%, and most preferably at least about 90-95% homology with any one of the amino acid sequences depicted

in Figures 2-5, and which qualitatively retains the laccase activity of the sequence described herein. Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have 5 been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be 10 interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to 15 the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different 20 industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. Such methods are described in, for example, Jin et al., *Holzforschung* 45(6): 467-468, 1991; US Patent No. 4,432,921; 25 EP 0 275 544; PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of 30 chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, *Current opinion in*

Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the

5 compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 10 92/01406, WO 92/18683, EP 0495836 and Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversiteit Gent. 56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem. 42: 273-282, 1991.

The laccase is particularly well-suited for use in hair dyeing. In such an application, the laccase is contacted with a dye precursor, preferably on the hair, whereby a controlled oxidation of the dye precursor is achieved to convert the precursor to a dye, or pigment producing compound, such as a quinoid compound. The dye precursor is 20 preferably an aromatic compound belonging to one of three major chemical families: the diamines, aminophenols (or aminonaphthols) and the phenols. The dye precursors can be used alone or in combination. At least one of the 25 intermediates in the copolymerization must be an ortho- or para-diamine or aminophenol (primary intermediate). Examples of such are found in Section V, below, and are also described in US Patent No. 3,251,742, the contents of which are incorporated herein by reference. In one embodiment, the starting materials include not only the enzyme and a 30 primary intermediate, but also a modifier (coupler) (or combination of modifiers), which modifier is typically a meta-diamine, meta-aminophenol, or a polyphenol. The modifier then reacts with the primary intermediate in the presence of the laccase, converting it to a colored

compound. In another embodiment, the laccase can be used with the primary intermediate directly, to oxidize it into a colored compound. In all cases, the dyeing process can be conducted with one or more primary intermediates, either 5 alone or in combination with one or more modifiers. Amounts of components are in accordance with usual commercial amounts for similar components, and proportions of components may be varied accordingly.

The use of this laccase is an improvement over the more 10 traditional use of H_2O_2 , in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with laccase can be conducted at alkaline, neutral or even acidic pH, and the oxygen needed for oxidation comes from the air, 15 rather than via harsh chemical oxidation. The result provided by the use of the *Polyporus* laccase is comparable to that achieved with use of H_2O_2 , not only in color development, but also in wash stability and light fastness. An additional commercial advantage is that a single 20 container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of H_2O_2 .

The present laccase can also be used for the polymerization of phenolic or aniline compounds present in 25 liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, *Fruit processing* 30 7/93, 248-252, 1993; Maier et al., *Dt. Lebensmittel-rindschau* 86(5): 137-142, 1990; Dietrich et al., *Fluss. Obst* 57(2): 67-73, 1990.

Laccases such as the *Polyporus* laccase are also useful in soil detoxification (Nannipieri et al., *J. Environ. Qual.*

20: 510-517, 1991; Dec and Bollag, Arch. Environ. Contam. Toxicol. 19: 543-550, 1990).

The invention is further illustrated by the following non-limiting examples.

5

EXAMPLES

I. ISOLATION OF A POLYPORUS PINISITUS LACCASE ENZYME

MATERIALS AND METHODS

1. Enzymatic assays

Unless otherwise stated, throughout the examples,

10 laccase activity is determined by syringaldazine and 2,2'-bisazino(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), as follows. The oxidation of syringaldazine is monitored at 530 nm with 19 μ M substrate. In 25 mM sodium acetate, 40 μ M cupric sulfate, pH 5.5, at 30°C, the activity is expressed 15 as LACU(μ mole/min). For pH profile studies, Britton & Robinson (B&R) buffers are used, and are prepared according to the protocol described in Quelle, Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964. ABTS oxidation is carried out with 1mM ABTS in 0.1 M NaAc, pH 5.0 20 at room temperature by monitoring either Δ Abs₄₀₅ in a 96-well plate (Costar) or Δ Abs₄₁₈ in a quartz cuvette. The overlay ABTS oxidase activity assay is carried out by pouring cooled ABTS-agarose (0.03-0.1 g ABTS, 1 g agarose, 50 ml H₂O, heated 25 to dissolve agarose) over a native IEF gel or PAGE and incubating at room temperature.

2. Initial isolation of laccase

In order to isolate the laccase, 800 ml of culture fluid is filtered by HFSC on a Supra filter (slow filtering). The clear filtrate is then concentrated and washed on an 30 Amicon cell with a GR81 PP membrane to a volume of 72 ml.

One ml aliquots of laccase are bound to a Q-sepharose HP (Pharmacia, Sweden) column, equilibrated with 0.1 M phosphate, pH7 and the laccase is eluted with a NaCl gradient. In all, 10 x 1 ml samples are purified, pooled,

concentrated and washed by ultrafiltration using a membrane with a molecular weight cut-off of 6kD.

3. Secondary purification

In a second purification, a fermentation broth is 5 filtered and concentrated by ultrafiltration. The starting material contains 187 LACU/ml. The concentrate is quick-filtered on a Propex 23 filter(P & S Filtration), with 3% Hyflo Cuper-Cel(HSC; Celite Corporation), followed by two 10 ultrafiltration on a Filtron filter with two membranes, each with a molecular weight cutoff of 3 kD. The resulting sample (2.5 mS/cm, pH 7.0, at 4°C) is applied to a 130 ml Q-Sepharose column, equilibrated with sodium phosphate, 1.1 mS/cm, pH 7.0. Under these conditions the laccase does not bind to the column, but elutes slowly from the column during 15 the application and wash with the equilibration buffer, resulting in a partial separation from other brownish material.

This partially purified preparation of 1.0mS, pH 7.0 at 20°C is applied to a Q-sepharose column. The column is 20 equilibrated with 20mM sodium phosphate, 2.2 mS, pH 7.0. Under these conditions, the laccase binds to the column and is eluted by a gradient of 0-1 M NaCl over 20 column volumes.

3. Sequencing

25 For internal peptide sequencing, the purified protein is digested with trypsin, followed by peptide purification with HPLC. Purified peptides are sequenced in an Applied Biosystems 473A sequencer.

B. RESULTS AND DISCUSSION

1. Initial characterization

Total yield of the initial purification is about 50 mg (estimated at A280nm). The purified enzyme has a rich blue color, and appears as only two very close bands on SDS-PAGE at about 65 kd. A native PAGE overlaid with substrate

shows that both bands have laccase activity with ABTS. The absorption spectrum shows that besides an absorption at A280nm, the purified laccase also shows absorption at about 600nm.

5 2. Sequencing

A N-terminal determination of the protein initially purified shows a single sequence:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn-Ala-Ala-Ala-Val-Ser-Pro-Asp-Gly-Phe-Pro...

10 Since the N-terminal sequence is not the ideal sequence for constructing a probe, additional experiments with a trypsin digest are conducted, followed by further purification(described above) and sequencing of fragments

2. Secondary purification and characterization

15 In the second purification, the second Q-Sepharose chromatographic step yields the following pools:

Q-Sepharose-2-pool-1 40 ml 112 LACU 47 LACU/A₂₈₀

Q-Sepharose-2-pool-3 80 ml 385 LACU 65 LACU/A₂₈₀

19 The elution yields >80% of the applied amount. The highly purified preparation Q-Sepharose-2-pool-3 has an A₂₈₀ = 5.9, and A₂₈₀/A₂₆₀ = 1.4. The purity of the laccase in the starting material is extremely high on a protein basis but the starting material is a very dark brown color. In SDS-PAGE, a double band is seen, with a dominating 65 kD band and a smaller 62 kD band. By anionic chromatography, only the dominating band is seen in the first peak(Q-Sepharose-2-pool-1), whereas both bands are seen in the second peak(Q-Sepharose-2-pool-3).

3. Sequence

30 A number of internal peptide sequences are determined, and compared with the *Coriolus hirsutus* (*Ch*) laccase sequence. The identified fragments are as follows:

Tryp 13:

Ser-Pro-Ser-Thr-Thr-Ala-Ala-Asp-Leu

Tryp 14:

Ser-Ala-Gly-Ser-Thr-Val-Tyr-Asn-Tyr-Asp-Asn-Pro-Ile-Phe Arg

Tryp 16:

Sequence 1:

5 Ser-Thr-Ser-Ile-His-Trp-His-Gly-Phe-Phe-Gln-Lys

Sequence 2:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn-Ala-Ala-Val

Tryp 18:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn

10 Tryp 19:

Sequence 1:

Leu-Gly-Pro-Ala-Phe-Pro-Leu-Gly-Ala-Asp-Ala-Thr-Leu-Ile-

Sequence 2:

Phe-Gln-Leu-Asn-Val-Ile-Asp-Asn-Asn-Thr-Thr-His-Thr-Met

15 Tryp 25:

Tyr-Ser-Phe-Val-Leu-Glu-Ala-Asn-Gln-Ala-Val-Asp-Asn-Tyr-Trp-

Ile-Arg

Tryp 27

Gly-Thr-Asn-Trp-Ala-Asp-Gly-Pro-Ala-Phe

20 II. ISOLATION OF A POLYPORUS PINISITUS LACCASE CDNA CLONE

A. MATERIALS AND METHODS

1. RNA preparation

RNA is isolated from 10 grams of *P. pinsitus* mycelium grown under xylidine induction for 6.5 hours, using the 25 guanidium/CsCl cushion method. The RNA is poly-A selected on an oligo-dT column, using standard conditions. 120 μ g mRNA is obtained and stored as lyophilized pellet in 5 μ g aliquots at -80°C.

2. Single stranded cDNA

30 Single stranded cDNA is synthesized using the reverse transcriptase "Super Script" (BRL) according to manufacturer's directions.

3. Construction of cDNA library

A cDNA library is constructed using the librarian IV cDNA kit (Invitrogen). Fifty cDNA pools, each containing approximately 5000 individual transformants, are obtained.

4. PCR

5 PCR is conducted under the following standard conditions: 100pmol of each primer, 10 μ l 10X PCR buffer (Perkin-Elmer), 40 μ l dNTP 0.5 mM, 2 μ l single stranded cDNA (or approximately 100 ng chromosomal DNA or 100 ng PCR fragment), H₂O to 100 μ l, 2.5U Taq polymerase. The cycles 10 are 3x(40°C/two minutes, 72°C/two minutes, 94°C/one minute) followed by 30x(60°C/two minutes, 72°C/two minutes, 94°C/1 minute).

B. RESULTS AND DISCUSSION

1. Cloning of *Polyporus pinsitus* laccase

15 PCR is carried out with the primer #3331:

ACCAGNCTAGACACACGGGNTC/AGATACTG/ACGNGAGAGCGGAC/TTGCTGGTC
ACTATCTTCGAAGATCTCG

and primer #3332:

CGCGGCCGCTAGGATCCTCACAATGGCCAA/CTCTCTG/CCTCG/ACCTTC.

20 A clear band of about 1500bp is obtained. The DNA is digested with NotI/HindIII, and fractionated on an agarose gel. The upper band (fragment #42) is purified and cloned into the *Aspergillus* vector pHd423. No transformants are obtained. Several attempts are carried out in order to 25 clone the fragment, including redigestion with the restriction enzymes, phosphorylation of the ends, filling in with klenow and blunt-end cloning in SmaI cut puC18, without success. Hybridization with a laccase probe based on the laccase described in Coll et al., *supra*, indicates that the 30 PCR product could be the *P. pinsitus* laccase. In a new attempt to clone the PCR fragment, a new PCR reaction is carried out, using the same conditions as for fragment #42. Again the result is a fragment of about 1500 bp (fragment #43). This time the fragment is cut with HindIII/BamHI, and

ligated to HindIII/BamHI-cut pUC18. Three clones, #43-/A,-B,-G are found to contain a fragment of 1500 bp. Partial sequencing reveals that these fragments are laccase related.

2. Expression of *Polyporus pinsitus* laccase

5 To express the laccase, the fragment #43 is joined to a signal sequence from a 43kD cellulase. The primer pH433 (TAGCGGATCCCACAATGCGTCCCTCCCCCTCCTCCCGTCCGCCGTTGTGGCCGCCCTG CCGGTGTTGGCCCTTGCCGGCATTGGGCCCGTCGGGAC) is used in a standard PCR reaction with a pUC forward primer (New England 10 Biolabs). All three clones are used as templates in order to minimize the risk of working with DNA containing errors.

The PCR generated DNA from the reaction with a primer pH433 and template 43-A and 43-G is cut with HindIII/BamHI and cloned into the *Aspergillus* expression vector

15 pH414 (described in detail below). Several transformants are obtained.

Clones pH433/43A-1,2, pH433/43G-2,-3 are transformed into *A. oryzae*. The transformants from each transformation (between 3-10) are analyzed for laccase production.

20 Activity is only obtained with pH433/43G-3. The positive transformants (numbers 1, 4, 6) are reisolated on amds plates, and retested. In an additional transformation round a further ten transformants are obtained with pH433/43G-3. The clones #20, 23, 26, 28, and 29 are positive. The clones 25 are reisolated and two single isolates are analyzed for laccase expression semiquantitatively by color development in an ABTS assay at pH 4.5. On a scale of +-++, several clones show moderate to strong expression of laccase.

Further cloning is conducted to identify a full length 30 clone. A xylidine-induced cDNA library consisting of approximately 350,000 transformants is screened using fragment #42-4 as a probe. More than 100 positive clones are detected. The clones are purified, rescreened, and analyzed on Southern blots. Two of the longest clones are

further characterized by DNA sequence determination. The longest clones are found to be identical and found to contain a poly-A stretch in the 3' end and to start at the amino acid number 4 in the amino terminus. A partial DNA 5 sequence is determined from different clones.

pHD433/43G-3 is then used in further cloning studies as described in the following Section IV.

III. PURIFICATION AND CHARACTERIZATION OF ADDITIONAL *POLYPORUS PINSITUS* LACCASE WILD-TYPE ENZYMES

10 A. MATERIALS AND METHODS

1. Culture conditions

Shake flasks (250 ml medium/2.8 l baffled flask) are inoculated with several agar plugs taken from a week-old PDA plate of *P. pinsitus*. The medium contains, per liter, 10 g glucose, 2.5 g L-asparagine, 0.2 g L-phenylalanine, 2.0 g 15 yeast extract, 2.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 2.0 ml AMG trace metals, 0.002 g CuSO₄·7H₂O, 1.0 g citric acid, made with tap water, pH 5.0 before autoclaving. The cultures are grown at 18-22°C on a rotary shaker with low agitation (~100 rpm). 20 After 7 days, the pH of each shake flask is adjusted to ~6.0 by the addition of 0.25 ml 5 N NaOH and the cultures are induced by adding 0.5 ml of a 2,5-xylidine stock solution (xylidine diluted 1:10 into ethanol) to each flask. Flasks are incubated for an additional 24 hours, at which 25 time the culture supernatant from each flask is recovered.

2. Materials.

Chemicals used as buffers are commercial products of at least reagent grade. Endo/N-glucosidase F is from Boehringer-Mannheim. Chromatography is performed on 30 Pharmacia FPLC. Spectroscopic assays are conducted on either a spectrophotometer (Shimadzu PC160) or a microplate reader (Molecular Devices).

3. Purification

Culture broth is filtered first on cheesecloth and centrifuged at 1000 x g to remove gelatinous pinkish xylidine polymer. The supernatant is then filtered on Whatman #2 paper and concentrated from 1500 to 250 ml on 5 S1Y100 (Amicon, Spiral concentrator) at 4°C. The concentrated broth is diluted with water until it reaches 0.8 mS (from 2.5 mS) and then concentrated on S1Y100 to 250 ml. The washed broth, thawed from -20°C freezing overnight, is subjected to Whatman #2 paper filtration to remove 10 residual pinkish material, and then pH adjusted by NaOH from pH 6.1 to pH 7.7. This yellowish broth, 275 ml with 0.8 mS, is applied on a Q-Sepharose XK-26 column (~64 ml gel) equilibrated with 10 mM Tris-HCl, pH 7.7, 0.7 mS. The first active laccase fraction runs through during loading and 15 washing by the equilibrating buffer. The elution is carried out by a linear gradient of 0-0.5 M NaCl in the equilibrating buffer over 8.8 bed-volume. The second and third active fractions are eluted around 0.15 and 0.35M NaCl, respectively. No more active fractions are detected 20 when the column is washed sequentially with 2 M NaCl and with 1 mM NaOH. The active fractions are pooled, adjusted to ~10mS, concentrated on Centricon-10 (Amicon), and then applied onto Superdex 75 (HR10/30, 24 ml, Pharmacia) equilibrated with 10 mM Tris-HCl, 0.15 M NaCl, pH 8, 14 mS. 25 During elution with the application buffer, laccase fractions are eluted off using the same elution volume for all three Q-Sepharose fractions, indicating very similar native molecular weight. The purity of the laccase is tested on SDS-PAGE.

30 4. Protein analysis

PAGE and native IEF are carried out on a Mini Protean II and a Model 111 Mini IEF cells (Bio-Rad). Western blots are carried out on a Mini trans-blot cell (Bio-Rad) with an alkaline phosphatase assay kit (Bio-Rad). The primary

antibodies are diluted 1000-fold during blotting. N-terminus sequencing is performed on an Applied Biosystems (ABI) 476A protein sequencer using liquid phase TFA delivery for cleavage and on-line HPLC for identification of PTH-

5 amino acids. Standard Fast Cycles and Pre-Mix Buffer System is used according to manufacturer's instructions.

Deglycosylation with glycosidase is done as follows: 3 μ g of protein and 3.6 units of glycosidase in 0.25M NaAc, pH 5, 20 mM EDTA, 0.05% 2-mercaptoethanol is incubated at 37°C for 18

10 hours with ovalbumin and bovine serum albumin serving as positive and negative control, respectively, and the mobility is detected by SDS-PAGE.

Amino acid analysis for determining extinction coefficients is done using Amino Quant 1090 HPLC system from

15 Hewlett Packard. Microwave facilitated vapor phase hydrolysis of lyophilized samples is done using the MDS-2000 hydrolysis-station (CEM, Matthews, NC). 6N HCl containing 1% phenol as a scavenger is used to create the acid vapors.

Hydrolysis time is 20 minutes at 70 psi (~148°C).

20 Hydrolyzed samples are lyophilized and redissolved in 20 μ l of 500pmol/ μ l sarcosine and norvaline as internal standards. 1 μ l is injected and analyzed according to manufacturer's instructions.

B. RESULTS AND DISCUSSION

1. Purification

The previously characterized *P. pinsitus* laccase has a pI of ~3.5. However, considerable laccase activity is detected in the run-through fraction of Q-Sepharose pre-equilibrated at pH 7.7. Upon a gradient elution, one more

30 active fraction comes off the column before the active fraction initially anticipated. UV-visible spectra and SDS-PAGE show that all three fractions contain mainly laccase. After further purification by gel filtration, different pI's under native non-denaturing conditions are detected for the

two new fractions and shown to be consistent with the elution order.

2. Characterization

The pure laccase preparations derived from Q-Sepharose 5 eluates behave as a rather well-defined band on SDS-PAGE at ~63 kDa. Deglycosylation detects ~14% w/w carbohydrates based on mobility change on SDS-PAGE. On native-IEF, the laccase preparations have bands of pI 6-6.5, 5-6.5, and 3.5. ABTS-agarose overlay show that all bands are active. Each 10 form in turn shows multiple isoforms under the IEF conditions.

The neutral and acidic forms have a typical UV-visible spectrum with maxima at 605 and 275 nm. The ratio of A_{275}/A_{605} is 30-40. The spectrum for the acidic-neutral form 15 has a peak at 276 nm and a shoulder around 600 nm.

The N-terminal sequencing shows that the neutral and neutral-acidic forms have the same first 29 residues (Table 1). The N-terminus of the acidic form matches 100% to that of the previously characterized form. All three forms 20 exhibit comparable cross-reactivity toward antibodies raised against previously characterized form.

Table 1. Structural and enzymatic properties of *P. pinsitus* laccases

<u>Form</u>	<u>N-terminus</u>	<u>LACU</u>	<u>$\Delta A_{405\text{min}}^{-1}(\text{ABTS})$</u>
5 Acidic	GIGPVA D LTITNAAVSPDGFSRQAVVVNG	92	4000
Acidic-	A*****(*)*VVA**P*****L*D*I****	75	4000
Neutral			
Neutral	A*****(*)*VVA**P*****L*D*I****	32	1000

10 *:Same residue as compared with the acidic form. (): weak signal

3. Laccase Activity

The specific activities (per A_{275}) of the three forms are tested by both ABTS and syringaldazine oxidations. The 15 shapes and optima of the pH activity profiles for the three forms are very close: all have optima at $\leq \text{pH} 4$ and pH 5-5.5 for ABTS and syringaldazine oxidations, respectively.

IV. ISOLATION OF MULTIPLE COPIES OF *POLYPORUS PINSITUS*

20 LACCASE ENZYMES AND GENES

A. MATERIALS AND METHODS

1. Strains

The following strains are employed in the methods described below: *E. coli* K802(*e14-(mrca)*, *mcrB*, *hsdR2*, 25 *galK2*, *galT22*, *supE44*, *metB1*; Clonetech); *E. coli* XL-1 Blue(*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac[F'proAB, lacIQZDM15, Tn10(tetr)]*; Stratagene) and *Polyporus pinsitus* CBS 678.70.

2. Genomic DNA isolation

30 Cultures of *P. pinsitus* are grown in 500 ml YG (0.5% yeast extract, 2% dextrose) at room temperature for 3 to 4 days. Mycelia are harvested through miracloth, washed twice with TE and frozen quickly in liquid nitrogen. The frozen mycelia are stored at -80°C. To isolate DNA, the mycelia

are ground to a fine powder in an electric coffee grinder. The powdered mycelia are resuspended in TE to a final volume of 22 ml. Four ml 20% SDS is added with mixing by inversion followed by incubation at room temperature for 10 minutes.

5 The sample is gently extracted with phenol:chloroform and centrifuged to separate the phases. The aqueous phase is collected and 400 μ l proteinase A(10 mg/ml stock) is added. The sample is incubated at 37°C for 30 minutes followed by a phenol:chloroform extraction. The aqueous phase is

10 precipitated by the addition of 0.1 volumes of 3 M Na acetate, pH 5.2 and 2.5 volumes 95% ethanol and freezing at 20°C for one hour. After centrifugation to precipitate the DNA, the pellet is resuspended in 6 ml TE, and 200 μ l boiled RNase A(10 mg/ml stock) is added. After incubation at 37°C,

15 100 μ l proteinase A(10 mg/ml stock) is added followed by incubation at 37°C for 30 minutes. The sample is phenol:chloroform extracted twice. To the aqueous phase, 0.1 volumes 3 M Na acetate and 2.5 volumes are added, and the sample is frozen at -20°C for 1 hour. Following

20 centrifugation, the pellet is gently resuspended in 400 μ l TE, and 40 μ l Na acetate and 1 ml 95% ethanol are added. The DNA is pelleted by centrifugation, and the pellet is washed in 70% ethanol. The final pellet is resuspended in 250 μ l TE.

25 3. RNA preparation

RNA is isolated from mycelia which are harvested from *P. pinisitus* cultures which are either induced for laccase expression by the addition of 2,5-xylidine or are uninduced. The mycelia are washed and frozen quickly in liquid N₂.

30 Frozen mycelia are ground to a fine powder in an electric coffee grinder. The powder is immediately suspended in 20 ml extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 50 mM EGTA, 0.8% tri-isopropyl naphthalene sulfonic acids, 4.8% p-aminosalicylic acid, pH 8.5). All solutions for RNA

extraction are made with diethylpyrocarbonate (DEP)-treated water. The sample is kept on ice and 0.5 volumes TE-saturated phenol:chloroform is added. The sample is mixed well by inversion for 2 minutes, and the phases are

- 5 separated by centrifugation. The aqueous phase is saved, and the organic phase is extracted with 2 ml extraction buffer and incubated at 68°C for 5 minutes. After centrifugation to separate the phases, the aqueous phases are pooled and extracted several time with phenol:chloroform
- 10 until there is no longer any protein at the interface. To the aqueous phase 0.1 volume 3 M Na-acetate, pH 5.2 and 2.5 volumes 95% ethanol are added to precipitate the RNA, and the sample is frozen at -20°C for 2 hours. The RNA is pelleted and resuspended in DEP water with RNase inhibitor.

15 4. DNA sequencing

Nucleotide sequences are determined using TAQ polymerase cycle sequencing with fluorescent-labeled nucleotides, and reactions are electrophoresed on an Applied Biosystems automatic DNA sequencer (Model 363A, version 20 1.2.0).

5. Preparation of genomic libraries

Two size-selected genomic libraries of *P. pinsitus* are constructed. A library of 5 to 6 kb BamHI fragments are constructed in pBluescript+. Genomic DNA is digested with 25 BamHI, and the digest is electrophoresed on a preparative agarose (IBI) gel. The region containing the 5 to 6 BamHI fragments is sliced from the gel. The DNA is isolated from the gel using a Geneclean kit (BIO 101). The DNA is ligated into pBluescript plasmid previously digested with BamHI and 30 dephosphorylated with BAP (GIBCO BRL). *E. coli* XL-1 Blue competent cells (Stratagene) are transformed with the ligation, and 12,000 white colonies are obtained.

A library of 7 to 8 kb BamHI/EcoRI fragments is constructed in pUC118. Ten µg genomic DNA is digested with

BamHI and EcoRI and treated with BAP(GIBCO BRL). Competent *E. coli* XL-1 Blue cells are transformed with the ligation, and the library contains ~8000 recombinants.

For the preparation of a total genomic library in lambda EMBL4, 25 μ g of *P. pinsitus* genomic DNA is partially digested with Sau3A. After digestion, the DNA is electrophoresed on a preparative low-melt agarose gel, and a band containing the 9 to 23 kb sized DNA is sliced from the gel. The DNA is extracted from the gel using β -agarose (New England Biolabs). The isolated EMBL4 arms (Clonetech) according to the supplier's directions. The ligation is packaged *in vitro* using a Gigapack II kit (Stratagene). The library is titered using *E. coli* K802 cells. The unamplified library is estimated to contain 35,000 independent recombinants. The library is amplified using *E. coli* K802 cells.

6. Southern and Northern Blots

DNA samples are electrophoresed on agarose gels in TAE buffer using standard protocols. RNA samples are electrophoresed on agarose gels containing formaldehyde. Both DNA and RNA gels are transferred to Zeta-Probe membrane(BIO-RAD) using either capillary action under alkaline conditions or a vacuum blotter. After transfer, the DNA gels are UV crosslinked. Blots are prehybridized at 65°C in 1.5X SSPE, 1% SDS, 0.5% non-fat dried milk and 200 μ g/ml salmon sperm DNA for 1 hour. Radioactive probes are added directly to the prehybridization solutions, and hybridizations are continued overnight at 65°C. Blots are washed with 2XSSC for 5 minutes at 65°C and with 0.2XSSC, 1%SDS, 0.1% Na-pyrophosphate at 65°C for 30 minutes twice.

Radioactive labeled probes are prepared using a α -³²P-dCTP and a nick translation kit(GIBCO-BRL).

7. Library screening

For screening of the size-selected 5-6 kb BamHI and 7-8 kb BamHI/EcoRI libraries ~500 colonies on LB carb plates and lifted the colonies to Hybond N⁺ filters (Amersham) using standard procedures. The filters are UV crosslinked

5 following neutralization. The filters are prehybridized at 65°C in 1.5X SSPE, 1% SDS, 0.5% non-fat dried milk, 200 µg/ml salmon sperm DNA for 1 hour. Nick-translated probes are added directly to the prehybridization solution, and hybridizations are done overnight at 65°C.

10 For screening of the genomic bank in EMBL, appropriate dilutions of the amplified library are plated with *E. coli* K802 cells on 100mM NZY top agarose. The plaques are lifted to Hybond N⁺ membranes (Amersham) using standard procedures. The DNA is crosslinked to the membranes using UV

15 crosslinking. The filters are prehybridized and hybridized using the same conditions as those mentioned above.

RESULTS AND DISCUSSION

1. Isolation of multiple copies of laccase gene

P. pinsitus genomic DNA is digested with several

20 different restriction enzymes for southern analysis. The blot is probed with the cDNA insert (isolated as a BamHI/SphI fragment from the pYES vector) which is labeled with α -P³²-dCTP. The blot is hybridized and washed as described above. The cDNA hybridizes to several restriction fragments for

25 most of the enzymes suggesting that there are multiple laccase genes in the genome. Because the cDNA hybridizes to a BamHI fragment of ~5.5 kb, a library of 5-6 kb BamHI fragments from *P. pinisitus* is constructed.

2. Screening of Genomic Libraries

30 The results from screening of the libraries are summarized in Table 2. The 5-6 kb BamHI size-selected library is screened with the original cDNA clone labeled with ³²P. Approximately 30,000 colonies are screened with hybridizations done at 65°C. Plasmid DNA is isolated from

two positive colonies and digested with BamHI to check for insert size. Both clones contain an ~5.5 kb BamHI insert. The cloned insert(LCC3) is sequenced from either end; the sequence has homology to the cDNA, but is clearly not the 5 cDNA encoded laccase. The partial DNA sequence of LCC3 also indicates that the LCC3 pUC118 clone does not contain the full gene.

From a southern blot of BamHI/EcoRI double digested DNA it is demonstrated that the cDNA hybridizes to an ~7.7 kb 10 fragment. A size-selected library in pUC118 is constructed containing 7-8 BamHI/EcoRI fragments. A total of ~8000 independent colonies are obtained and screened by hybridization with a ³²P labeled insert. Plasmid DNA is isolated from the positive colonies and digested with BamHI 15 and EcoRI. Restriction analysis of the plasmids demonstrate that they fall into two classes. One class (LCC4) contains four clones which are all identical and have an ~7.7 kb BamHI/EcoRI insert which hybridizes to the cDNA. A second class(LCC1) contains two clones which are identical and have 20 inserts of ~7.2 kb which hybridize to the cDNA. Partial DNA sequencing of clones LCC1 and LCC4 demonstrate that clone 21 is the genomic clone of the original cDNA, while LCC4 codes for another laccase. The partial DNA sequence of LCC1 shows that the pUC118 clone does not contain the full gene and 25 that a fragment upstream of the EcoRI site is needed.

At the same time the size selected 7-8 BamHI/EcoRI library is being constructed, a *P. pinisitus* genomic bank in EMBL4 is constructed containing ~35,000 independent recombinant phage. Ten positive plaques are picked and 30 purified. DNA is isolated from the purified phage lysates. Restriction digests of EMBL DNAs demonstrates that there are three classes of clones. The first class(11GEN) is defined by two sibs whose inserts contain a BamHI/EcoRI fragment of the same size as LCC1 which hybridizes to the LCC1 insert.

The second class(12GEN) contains one clone which has a different restriction pattern than the 11GEN class and whose insert contains a different restriction pattern than the 11GEN class and whose insert contains an ~5.7 kb BamHI/EcoRI 5 fragment. The third class is defined by a single clone whose insert contains an ~3.2 kb BamHI/EcoRI fragment which hybridizes to the LCC1 insert. DNA sequence analysis demonstrates that clone 11GEN contains the LCC1 BamHI/EcoRI fragment and both 5' and 3" flanking regions. It is also 10 demonstrated that clone 12GEN contains a portion of the LCC1 insert.

The *P. pinisitus* EMBL genomic bank is also screened with the LCC3 BamHI insert in order to clone the full gene. Approximately 30,000 plaques are plated and lifted from 15 hybridization. Five plaques which hybridize to the LCC3 (BamHI/EcoRI) insert are identified and purified. DNA is isolated from the purified phage stocks. Southern analysis of *P. pinisitus* genomic DNA demonstrates that the LCC3 BAMHI insert hybridizes to an ~7kb EcoRI fragment. 20 Restriction digests and southerns demonstrate that 4 of the clones contain restriction fragments which hybridize to the EcoRI/BamHI(1.6 kb) fragment and that the clones fall into three classes. Class one is defined by a single clone(LCC5) whose insert contains a 3kb EcoRI fragment which hybridizes 25 to the LCC3 BamHI/EcoRI fragment. Another class is defined by clone(LCC2) whose insert contains an ~11 kb EcoRI fragment which hybridizes to the LCC3 BamHI/EcoRI insert. The third class is defined by two clones which are not identical but contain many of the same restriction 30 fragments; these clones both contain an ~7.5 kb EcoRI fragment which hybridizes to the LCC3 insert. Further analysis of this third class indicates that they are identical to clone LCC4. Partial DNA sequencing of LCC5 and LCC2 indicates that both of these clones code for laccases;

however, neither is identical to any of the above mentioned laccase genes (LCC1, LCC3, or LCC4). At this point, five unique laccase genes are cloned; however, the fragments subcloned from LCC5 and LCC2 do not contain the full genes.

- 5 From the DNA sequencing of the 3 kb EcoRI fragment from clone LCC5 it is determined that ~200 base pairs of the N-terminus are upstream of the EcoRI site. A 380 bp EcoRI/MluI fragment from LCC5 is used to identify for subcloning a MluI fragment from the LCC5 EMBL clone. An
- 10 ~4.5 MluI fragment from the LCC5 EMBL clone is subcloned for sequencing and shown to contain the N-terminal sequence.

To clone the N-terminal half of the LCC3 laccase gene, the *P. pinsitus* genomic bank is probed with an ~750 bp BamHI/StuI restriction fragment from the LCC3 pUC118 clone.

- 15 Approximately 25,000 plaques are screened and five plaques appear to hybridize with the probe. Upon further purification only three of the clones are still positive. Two of the clones give very strong signals and the restrictions digests of DNA isolated from these phage
- 20 demonstrate that both contain an ~750 bp BamHI/StuI fragment in their inserts and that the two clones are not identical but overlapped. Based on results of Southern analysis, an ~8.5 kb fragment from these clones are subcloned for sequencing. The EcoRI fragment is shown to contain the
- 25 entire gene.

To clone the N-terminal half of the LCC2 laccase gene, the *P. pinsitus* genomic bank in EMBL4 is probed with an ~680 bp EcoRI/PvuI of the EMBL LCC2 clone. Thirty thousand plaques are screened by hybridization at 65°C, and 15

- 30 plaques appear to hybridize with the probe. All fifteen are purified, and DNA is isolated. The clones can be placed in four classes based on restriction patterns. Seven of the clones are all sibs, and are identical to the original EMBL clone of LCC2. The second class is defined by 3 clones

which are sibs. An ~4 kb HindIII fragment is subcloned from this class for sequencing and is shown to contain the N-terminal half of LCC2. A third class is defined by a single clone and is not characterized further.

5 3. DNA sequencing

The complete DNA sequences of the five genomic clones is determined as described in Materials and Methods.

Sequencing of clone LCC2 demonstrate that it probably codes

for the second form of laccase(neutral pI) isolated from

10 culture broth from an induced *P. pinsitus* culture as described above. The N-terminal protein sequence from the neutral pI laccase and the predicted N-terminus for the protein coded for by LCC2 are compared, and show identity.

15 The predicted pI for the protein coded for by clone LCC2 is 5.95, which is in good agreement with the experimental pI determined for the second form of laccase being between 5.0 and 6.5. Figures 1-5 (SEQ ID NOS. 1-5) show the DNA sequences and predicted translation products for the genomic clones. For LCC1, the N-terminus of the mature protein as

20 determined by protein sequencing and predicted by Von Heijne rules is Gly at position 22. The N-terminus is Gly-Ile-Gly-Pro-Val-Ala-. For LCC2 the N-terminal amino acid of the mature protein as determined by protein sequencing is Ala at position 21. The N-terminus is Ala-Ile-Gly-Pro-Val-Ala-.

25 For LCC3 the predicted N-terminal amino acid of the mature protein is Ser at position 22, with the N terminus being Ser-Ile-Gly-Pro-Val-Thr-Glu-Leu-. For LCC4, the predicted N-terminal amino acid is Ala at position 23 with the N-terminus being Ala-Ile-Gly-Pro-Val-Thr-. For LCC5 the

30 predicted N-terminal amino acid is Ala at position 24 with the N-terminus being Ala-Ile-Gly-Pro-Val-Thr-Asp. A comparison of the structural organization of the genes and the predicted proteins they code for is presented in Table 1. It will be seen that the five genes have different

structural organizations and code for proteins of slightly different sizes. Comparisons between the predicted proteins of the genomic clones and other fungal laccase are also done. Table 2 shows a comparison of the predicted laccase 5 to each other and to other fungal laccases. Clone LCC1 (the induced laccase first characterized) has the most identity (90%) to the *Coriolus hirsutus* laccase and the PM1 basidiomycete laccase (Coll et al., *supra*). The other four laccases have between 64 and 80% identity to the *C. hirsutus* 10 laccase. The laccase coded for by LCC3 has the least identity to the LCC1 laccase and the other fungal laccases shown in Table 2. LCC2 appears to be the second wild-type laccase isolated as described above; based on the N-terminal sequences of the isolated clones, it also appears that the 15 "neutral" and acidic "neutral" wild-type laccases are the same enzyme which is encoded by the LCC2 sequence.

Table 1 Comparison of Structural Organization and Predicted Proteins of the *P. pinsitis* Genomic Clones.

<u>Gene</u>	<u># Introns</u>	<u>Size of Predicted Precursor Protein</u>	<u>Size of Predicted Mature Protein</u>	<u>Predicted Isoelectric Point</u>
21GEN	8	520	499	4.49
23GEN	10	519	498	5.95
24GEN	12	516	495	5.23
31GEN	11	510	488	4.06
41GEN	11	527	504	4.07

Table 2 Amino Acid Identity Between *P. pinsitidis* Laccases and Other Fungal Laccases.

	21GEN	23GEN	24GEN	31GEN	41GEN	CRIPHA	CRIPHE	PBILAC	PM1
21GEN	<u>79%</u>	79%	64%	70%	72%	90%	91%	64%	80%
23GEN	79%	<u>65%</u>	65%	66%	69%	80%	81%	62%	74%
24GEN	64%	65%	<u>61%</u>	61%	65%	64%	65%	61%	63%
31GEN	70%	66%	61%	<u>75%</u>	75%	69%	70%	64%	69%
41GEN	72%	69%	65%	75%	<u>71%</u>	71%	72%	64%	71%
CRIPHA	90%	80%	64%	69%	71%	<u>—</u>	99%	64%	80%
CRIPHE	91%	81%	65%	70%	72%	99%	<u>—</u>	65%	81%
PBILAC	64%	62%	61%	64%	64%	64%	65%	<u>—</u>	65%
PM1	80%	74%	63%	69%	71%	80%	81%	65%	<u>—</u>

21GEN, 23GEN, 24GEN, 31GEN and 41GEN= *P. pinsitidis* laccase clones

CRIPHA= *Coriolus hirsutus* laccase A

CRIPHE= *C. hirsutis* laccase B

PBILAC= *Phlebia radiata* laccase

PM1= Basidiomycete PM1 laccase (CECT2971)

5. Northern blots

RNA is isolated from mycelia from both a xylidine-induced culture and an uninduced culture. RNA is blotted to membrane after electrophoresis, and the blot is probed with 5 the cDNA insert, or a small fragment containing ~100 bp of the 23GEN promoter and the first 100 bp of the coding region. A transcript of about 1.8 kb hybridizes to both the induced and uninduced RNA samples; however, transcription of this message is clearly induced by the addition of xylidine 10 to the culture.

III. EXPRESSION OF *P. PINSITUS* LACCASE IN *ASPERGILLUS*

MATERIALS AND METHODS

1. Strains

A. oryzae A1560, A. oryzae HowB104 (fungamyl delete, 15 pyrg), A. oryzae HowB101pyrg, A. niger Bo-1, A. niger Bo-80, A. niger ATCC1040, A. niger NRRL337, A. niger NRRL326, A. niger NRRL326, A. niger NRRL2295, A. niger ATCC11358, A. niger NRRL322, A. niger AT10864, A. japonicus A1438, A. phoenicis, A. foetidus N953.

2. Media

For the shake flask cultivation of the A. niger, A. foetidus, and A. phoenicis MY50 (per liter: 50 g maltodextrin, 2 g MgSO₄·H₂O, 10 g KH₂PO₄, 2 g K₂SO₄, 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 2 g urea, pH 25 6.0) media is used. For the shake flask cultivation of the A. oryzae A1560 and HowB101 strains MY51 (per liter: 30 g maltodextrin, 2 mg MgSO₄, 10 g KH₂PO₄, 2 g K₂SO₄, 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 1 g urea, 2 g (NH₄)₂SO₄, pH 6.0) is used. For the shake flask analysis of 30 the A. oryzae HowB104 strains, MY51 maltose (same as MY51 but with 50g of maltose instead of maltodextrin) media is used. For the shake flask analysis of the A. japonicus strains M400 media (per liter: 50 g maltodextrin, 2 g MgSO₄, 2 g

KH_2PO_4 , 4 g citric acid, 8 g yeast extract, 0.5 ml trace metals, 2 g urea, pH 6.0.

Cultures grown overnight for protoplast formation and subsequent transformation are grown in YEG(0.5% yeast extract, 2% dextrose). For strains that are *pyrg*, uridine is supplemented to 10 mM final concentration.

3. Screening for laccase production

Primary transformants are screened first on a minimal medium plates containing 1% glucose as the carbon source and 10 1mM ABTS to test for production of laccase. Transformants that give green zones on the plates are picked and spore purified before shake flask analysis is done.

Shake flask samples are centrifuged to clear the broth. Dilute or undiluted broth samples are assayed with ABTS

15

RESULTS AND DISCUSSION

1. Expression in shake flasks

The first expression vector constructed is pDSY1, which contains the TAKA promoter, TAKA signal sequence, P . 20 *pinisitus* laccase cDNA beginning at the mature N-terminus and the AMG terminator. The TAKA signal sequence: laccase insert is constructed in 2 steps. First by site directed mutagenesis, an AgeI site beginning at bp 107 of the laccase mature coding region is created by a single base change and 25 a NsII site is created ~120 bp downstream of the laccase stop codon(ACG GGT->ACC GGT and TTC GCT->ATG CAT, respectively). A small PCR fragment beginning with an SfiI site and ending with the AgeI site at 107 bp in laccase is PCR amplified. This fragment contains a piece of the TAKA 30 signal sequence and the first ~107 bp of the mature laccase cDNA. Further DNA sequencing of this fragment shows it has a single base change that leads to a substitution of Asn for Thr at position 9 in mature laccase. This substitution creates a potential N-linked glycosylation site. The PCR

fragment and AgeI/NsiI fragments are cloned into pMWR1 (Figure 6) which has been digested with SfiI/NsiI. The vector pMWR1 contains the TAKA promoter, a portion of the TAKA signal sequence which ends with an SfiI site, and the 5 TAKA terminator with a NsiI site inserted directly 5' to the terminator. The resulting expression vector (Figure 7) is used to cotransform several hosts. Methods for co-transformation of *Aspergillus* strains are as described in Christensen et al., *supra*.

10 In the second laccase expression vector, the base change in DSY1 which leads to the substitution of Asn for Thr at amino acid 9 is reverted back to wild type by a PCR reaction. The second expression vector pDSY2 is identical to pDSY1 except for this single base change. Three 15 different *A. oryzae* strains and several *A. niger* strains are cotransformed with pDSY2 and either pTOC90 (WO 91/17243) which carries the *A. nidulans* *amdS* gene or pSO2 which carries the *A. oryzae* *pyrG* gene.

Expression of laccase is observed in all hosts tested, 20 with both DSY1 and DSY2. Yields range from 0.1-12.0 $\Delta_{abs}/min/ml$, with highest yields being observed with *A. niger* strains.

A construct pDSY10 is made which contains the TAKA 25 promoter, laccase full-length cDNA including its own signal sequence and the AMG terminator. A 200 bp BamHI/AgeI fragment which has a BamHI site immediately 5' to the ATG of the initiation codon and an AgeI site at the same position as in pDSY1 is PCR amplified using *lac1* as template. A 30 MluI/HindIII fragment is PCR amplified using pDSY2 as template and begins with the MluI site present in the cDNA and ends with a HindII site directly 3' to the stop codon of laccase. The above two fragments and the AgeI/MluI fragment

from pDSY2 are ligated into pHD414 to yield pDSY10 (Figure 8).

The vector pHD414 used in expression of laccase is a derivative of the plasmid p775 (EP 238 023). In contrast to 5 this plasmid, pHD414 has a string of unique restriction sites between the TAKA promoter and the AMG terminator. The plasmid is constructed by removal of an approximately 200 bp long fragment (containing undesirable RE sites) at the 3' end of the terminator, and subsequent removal of an 10 approximately 250 bp long fragment at the 5' end of the promoter, also containing undesirable sites. The 200 bp region is removed by cleavage with NarI (positioned in the pUC vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase + 15 dNTP, purification of the vector fragment on a gel and religation of the vector fragment. This plasmid is called pHD413. pHD413 is cut with StuI (positioned in the 5' end of the promoter) and PvuII (in the pUC vector), fractionated on gel and religated, resulting in pHD414. Cotransformation 20 of *A. oryzae* HowB104 and *A. niger* Bo-1 are done using pToC90 for selection. Yields in shake flask are comparable to those seen with pDSY2.

2. Expression in fermentors

A 1 ml aliquot of a spore suspension of *Aspergillus* 25 *niger* transformant Bo-1-pDSY10-4 (approximately 10⁹ spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (glucose, 75g/l; soya meal, 20 g/l; MgSO₄·7H₂O, 2g/l; KH₂PO₄, 10g/l; K₂SO₄, 2g/l; CaCl₂·2H₂O 0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; 30 trace metals [ZnSO₄·7H₂O, 14.3 g/l; CuSO₄·5H₂O, 2.5 g/l; NiCl₂·6H₂O, 0.5 g/l; FeSO₄·7H₂O, 13.8 g/l, MnSO₄·H₂O, 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18

hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media (maltodextrin MD01 300 g/l; $MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4 , 2g/l; citric acid 2g/l; K_2SO_4 , 2.7 g/l; $CaCl_2 \cdot 2H_2O$, 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l; made with tap water and pH adjusted to 6.0 before autoclaving). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitation rate is maintained at 800 rpm for the first 24 hours after inoculation and at 1300 rpm for the remainder of the fermentation. The pH of the fermentation is kept at 4.0 by the automatic addition of 5N $NaOH$ or H_3PO_4 . Sterile feed (urea, 50 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 40 g of feed is added initially before inoculation; after inoculation, feed is at a constant rate of 2.5 g/l h.

Copper is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank to a final level of 0.5 mM. Samples for enzyme activity determination are withdrawn and filtered through Miracloth to remove mycelia. These samples are assayed for laccase activity by a LACU assay. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 55 LACU/ml is achieved after 190 hours. This corresponds to approximately 350mg/l of recombinant laccase expressed.

30 IV. PURIFICATION OF RECOMBINANT LACCASE

MATERIALS AND METHODS

1. Materials.

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Endo/N-glycosidase G is

from Boehringer-Mannheim. Chromatography is performed on either a Pharmacia's FPLC or a conventional open column low pressure system. Spectroscopic assays are conducted on a Shimadzu PC160 spectrophotometer.

5 2. Purification

 (a) DSY2

2.8 liters cheese-cloth filtered broth(pH 7, 19mS) obtained from an *A. oryzae* pDSY2 transformant as described above is filtered on 0.45 μ Corning filter and concentrated 10 on Spiral Concentrator(Amicon) with S1Y30 membrane to 200ml. The concentrate pH is adjusted to 7.5, diluted with 4.8 l water to achieve 1.2 mS, and concentrated on S1Y30 to 200ml. 50ml of this broth solution is applied onto a Q-Sepharose column(XK16, 34ml gel), pre-equilibrated with 10mM Tris, pH 15 7.5, 0.7 mS(Buffer A). The blue laccase band that migrates slowly during loading is eluted by a linear gradient of Buffer B(Buffer A plus 0.5 M NaCl). 24 ml of pooled laccase fractions are concentrated on Centricon-100(Amicon) to 4.5 ml and applied onto a Superdex 200 column(HiLoad 16/60, 120 20 ml gel). During the development with Buffer C(Buffer A plus 0.15 M NaCl, 14.4 mS), the blue laccase fractions elute followed by brownish contaminant fractions. Only the first half of the elution band(detected by Abs_{600}) show a high laccase to contaminant ratio and are pooled. The pooled 25 fractions are dialyzed in 10mM Bis-Tris, pH 6.8, 0.6mS(Buffer D), applied onto a Mono-Q column(Mono-Q 5/5, 1ml) equilibrated with Buffer D, and eluted with Buffer E(Bufer D plus 0.5 M NaCl) using a linear gradient. The laccase fractions, which come out round 27% Buffer E, are 30 pure as judged by SDS-PAGE. At each step, the laccase fractions are routinely checked by ABTS oxidation, SDS-PAGE, and Western Blot.

 (b) DSY10

2.8 liters cheese-cloth filtered broth(pH 7.3, 24mS) obtained from HowB104-pDSY10 is filtered on Whatman #2 paper and concentrated on Spiral Concentrator(Amicon) with S1Y100 membrane to 210ml. The concentrate pH is diluted with 5 water to achieve 1.2 mS, and concentrated on S1Y100 to 328 ml. This broth solution is applied onto a Q-Sepharose column(XK26, 120 ml gel), pre-equilibrated with 10mM Tris, pH 7.5, 0.7 mS(Buffer A). The blue laccase band that migrates slowly during loading is eluted by a linear 10 gradient of Buffer B(Buffer A plus 2 M NaCl). 120 ml of pooled laccase fractions are diluted with water to achieve 1.1mS and then concentrated on S1Y100 to 294 ml and applied onto a Mono-Q column(HiLoad 16/10, 40 ml gel) pre-equilibrated with Buffer A. The laccase slowly passes 15 through the column during loading and washing with Buffer A. The pooled fractions which have a pH reading of 5.6, are loaded on a Mono-Q column(HiLoad 16/10, 40 ml gel), pre-equilibrated with Buffer C(10mM MES, pH 5.5, 0.1 mS). The laccase fractions elute by a very shallow gradient of Buffer 20 D(Buffer C + 1M NaCl). Enzymatic assays are conducted as described above.

3. Protein analysis

Total amino acid analysis, N-terminal sequencing, deglycosylation, SDS-PAGE, IEF, and Western blots are 25 performed as decribed above.

B. RESULTS AND DISCUSSION

1. Purification and Characterization

Overall a 256-fold purification and a yield of 37% are achieved for DSY10, and a 246-fold purification and a yield 30 of 14% are achieved for DSY2 In terms of electrophoretic pattern, spectral properties and activity, purified DSY2 and DSY10 are indistinguishable. Purified recombinant laccases behave as a dimer on gel filtration, and exhibit subunit molecular weight which is somewhat larger than that of the

wild type laccase, indicating a post-translational processing in *A. oryzae* that results in the extra glycosylation on the recombinants. Deglycosylation has confirmed the difference in mass arising from extra 5 sugars (Table 3).

Table 3. Molecular and spectral properties of recombinant and wild-type laccase

5	MW, kDa		Carbohydrate	PI	λ_{max} , nm (ϵ , l/g*cm)
	Native	subunit	w/w%		
WT	~130	~63	~7	3.5	275(1.8) 615(0.12)
Rec.	~130	~67	~13	3.5	275(1.7) 615(0.11)

10

The spectra of the purified laccases have maxima of 615 nm and 275, with the ratio of absorbance at 275 nm to that at 615 nm being 16, indicating one Type I Cu per subunit. The ratio of absorbance at 330nm to that at 615nm is 1.0, close 15 to the 0.75 value of *Rhus vernicefera* laccase, suggesting the presence of one Type II and two Type III copper ions per subunit. The extinction coefficient determined by amino acid analysis is 1.71/(g*cm),

3. Activity

20

The laccase activity is measured by syringaldazine and ABTS oxidations. Expressed per A₂₇₅, the laccase has a value of 83 for LACU. Expressed per mg, it has a LACU of 141. The pH profile of the laccase is provided in Figure 9.

25

V. USE OF POLYPORUS LACCASE TO DYE HAIR

The dyeing effect of *Polyporus pinsitus* laccase is tested and compared to the dyeing effect of 3% H₂O₂ on various dye precursors (listed below) and further on 0.1% p-phenylenediamine compared with a number of modifiers.

30

Materials:

Dye precursors:

0.1 % p-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0. (pPD)

0.1 % p-toluylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % chloro-p-phenylenediamine in 0.1 M K-phosphate buffer, pH 7.0.

5 0.1 % p-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % o-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 3,4-diaminotoluene in 0.1 M K-phosphate, buffer pH 7.0.

10 Modifiers:

0.1 % m-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 2,4-diaminoanisole in 0.1 M K-phosphate buffer, pH 7.0.

15 0.1 % α -naphthol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % hydroquinone in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % pyrocatechol in 0.1 M K-phosphate buffer, pH 7.0.

0.1% resorcinol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 4-chlororesorcinol in 0.1 M K-phosphate buffer, pH 20 7.0.

When a modifier is used, the dye precursor p-phenylene-diamine is combined with one of the above indicated modifiers so that the final concentration in the dyeing 25 solution is 0.1 % with respect to precursor and 0.1 % with respect to modifier. The enzyme used is a recombinant laccase from *Polyporus pinisitus*, at a concentration of 10 LACU/ml.

30 Other solutions used in the process are 3% H_2O_2 (in the final dye solution), and a commercial shampoo.

The quantitative color of the hair tresses is determined on a Datacolor Textflash 2000 (CIE-Lab) by the use of

CIE-Lab parameters L* ("0"=black and "100"=white) combined with a* ("-"=green and "+"=red). DL* and Da* are the delta values of L* and a*, respectively, of a sample when compared to L* and a* of untreated hair. The Light fastness is 5 determined under a day light bulb (D65) at 1000 LUX.

Hair tresses of blond European hair (1 gram) are used. 4 ml dye precursor solution (including modifier) is mixed with 1 ml laccase or 1 ml H₂O₂ on a Whirley mixer, applied to 10 the hair tresses and kept at 30°C for 60 minutes. The hair tresses are then rinsed with running water, combed, and air dried.

The results of the dyeing effect test are displayed below in 15 Table 4-6 and further in the graphs in Figures 10 to 12.

Table 4

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
1	p-phenylenediamine (Reference)	62.85	4.03	-9.41	1,61
2	p-phenylenediamine + Laccase	28.70	0.33	-43.56	-2,10
3	p-phenylenediamine + 3% H ₂ O ₂	21.88	2.04	-50.37	-0,39
4	p-Toluylenediamine (Reference)	58.14	4.34	-14.11	1.92
5	p-Toluylenediamine + Laccase	36.70	8.09	-35.56	5.67
6	p-Toluylenediamine + 3% H ₂ O ₂	42.30	6.24	-29.95	3.81
7	chloro-p-phenylenediamine (Reference)	69.82	3.23	-2.43	0.81
8	chloro-p-phenylenediamine + Laccase	35.58	9.36	-36.68	6.93
9	chloro-p-phenylenediamine + 3% H ₂ O ₂	45.42	9.59	-26.84	7.17
10	p-aminophenol (Reference)	66.62	5.03	-5.63	2.61
11	p-aminophenol + Laccase	42,42	7.38	-29,84	4.95
12	p-aminophenol + 3% H ₂ O ₂	50.54	9.42	-21.72	7.26
13	o-aminophenol (Reference)	69.39	4.82	-2.89	2.39
14	o-aminophenol + Laccase	60.20	12.92	-12.05	10.50
15	o-aminophenol + 3% H ₂ O ₂	63.49	10.38	-8.77	7.96
16	3,4-diaminotoluene (Reference)	69.62	3.57	-2.63	1.15
17	3,4-diaminotoluene + Laccase	39.51	3.15	-32.74	0.73
18	3,4-diaminotoluene + 3% H ₂ O ₂	59.32	4.16	-12.94	1.74

L*: 0=black, 100=white a*: -=green, + =red

Table 5

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
19	p-phenylenediamine + m-phenylenediamine (Reference)	58.82	0.43	-13,44	-1,99
20	p-phenylenediamine + m-phenylenediamine + Laccase	27.20	0.83	-45,05	-1,59
21	p-phenylenediamine + m-phenylenediamine + 3% H ₂ O ₂	16.96	0.13	-55,29	-2,59
22	p-phenylenediamine + 2,4 - diaminoanisole (Reference)	35.37	-0.02	-36,89	-2,45
23	p-phenylenediamine + 2,4 - diaminoanisole + Laccase	24.56	2.99	-47,70	0,57
24	p-phenylenediamine + 2,4-diaminoanisole + 3% H ₂ O ₂	15.06	2.21	-57,20	-0,21
25	p-phenylenediamine + α -naphthol (Reference)	54.33	2.54	-17,93	0,12
26	p-phenylenediamine + α -naphthol + Laccase	29.53	4.03	-42,72	1,60
27	p-phenylenediamine + α -naphthol + 3% H ₂ O ₂	19.58	3.90	-52,68	1,47
28	p-phenylenediamine + hydroquinone (Reference)	53.25	4.08	-19,01	1,65
29	p-phenylenediamine + hydroquinone + Laccase	40.48	5.00	-31,77	2,58
30	p-phenylenediamine + hydroquinone + 3% H ₂ O ₂	29.06	4.96	-43,20	2,53

L*: 0=black, 100=white a*: -=green, + =red

Table 6

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
31	p-phenylenediamine + pyrocatechol (Reference)	53.78	1.68	-18.47	-0.74
32	p-phenylenediamine + pyrocatechol + Laccase	30.77	2.64	-41.49	0.22
33	p-phenylenediamine + pyrocatechol + 3% H ₂ O ₂	22.15	3.30	-50.11	0.88
34	p-phenylenediamine + resorcinol (Reference)	62.12	4.23	-10.14	1.81
35	p-phenylenediamine + resorcinol + Laccase	36.14	2.91	-36.11	0.49
36	p-phenylenediamine + resorcinol + 3% H ₂ O ₂	23.94	3.16	-48.31	0.74
40	p-phenylenediamine + 4-chlororesorcinol (Reference)	61.18	4.70	-11.07	2.28
41	p-phenylenediamine + 4-chlororesorcinol + Laccase	36.00	2.76	-36.26	0.34
42	p-phenylenediamine + 4-chlororesorcinol + 3% H ₂ O ₂	22.63	2.60	-49.63	0.18

L*: 0=black, 100=white a*: -=green, +=red

The oxidative hair dyeing is carried out as described above, except that 50 LACU/ml *Polyporus pinsitus* laccase was used.

To test wash stability, the dyed hair tresses are wetted and washed for 15 seconds with 50 μ l of commercial shampoo, and rinsed with water for 1 minute. The hair tresses are washed up to 20 times.

The results of the hair wash test are displayed in figure 13. It can be seen in figure 13 that the wash stability of hair washed up to 20 times is excellent, when 10 using *Polyporus pinsitus* laccase for oxidative dyeing.

To test light fastness, tresses of blond european hair are used for testing the light fastness of hair dyed using *Polyporus pinsitus* laccase in comparison to hair dyed using H_2O_2 . p-phenylene-diamine is the dye precursor. The dyeing of 15 the hair is carried out as described above. One hair tress is kept dark, while an other is kept at day light (i.e. under a day light bulb (D65)), at approximately 1000 LUX) for up to 275 hours. The CIE-Lab-values are determined immediately after the dyeing of the hair, and further during 20 exposure to day light.

The results of the test are displayed in figure 14. Figure 14 shows that the hair dyed with p-phenylene-diamine using *Polyporus pinsitus* laccase has the same light fastness as hair dyed using H_2O_2 .

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Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural 30 Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria,

Illinois, 61604 on May 25, 1994 and given the following accession numbers.

<u>Deposit</u>	<u>Accession Number</u>
<i>E. coli</i> DH5 α containing	NRRL B-21263
5 pDSY22(41GEN; an ~3.0 kb EcoRI insert)	
<i>E. coli</i> DH5 α containing	NRRL B-21268
pDSY23(41GEN; an ~4.5 kb MluI insert; insert contains a small portion of the EcoRI fragment of pDSY22 and sequences	
10 5' to the EcoRI fragment)	
<i>E. coli</i> XL-1 Blue containing	NRRL B-21264
pDSY21(31GEN; an ~7.7 kb EcoRI/BamHI insert)	
<i>E. coli</i> XL-1 Blue containing	NRRL B-21265
15 pDSY18(21GEN; an ~8.0 kb BamHI insert)	
<i>E. coli</i> DH5 α containing	NRRL B-21266
pDSY19(23GEN; an ~4 kb HindIII insert)	
<i>E. coli</i> DH5 α containing	NRRL B-21267
pDSY20(24GEN; an ~8.5 kb EcoRI insert)	

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Novo Nordisk Biotech, Inc.
- (B) STREET: 1445 Drew Avenue
- (C) CITY: Davis, California
- (D) COUNTRY: United States of America
- (E) POSTAL CODE (ZIP): 95616-4880
- (F) TELEPHONE: (916) 757-8100
- (G) TELEFAX: (916) 758-0317

(i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Alle
- (C) CITY: Bagsværd
- (D) COUNTRY: Denmark
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- (G) TELEFAX: +45 4449 3256
- (F) TELEX: 37304

(ii) TITLE OF INVENTION: PURIFIED POLYPOROUS LACCASES AND
NUCLEIC ACIDS ENCODING SAME

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Novo Nordisk of North America, Inc.
- (B) STREET: 405 Lexington Avenue, Suite 6400
- (C) CITY and STATE: New York, New York
- (D) COUNTRY: U.S.A.
- (E) ZIP: 10174-6401

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: to be assigned
- (B) FILING DATE: 15-June-1995

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/265,534
- (B) FILING DATE: 24-June-1994

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Lowney, Karen A.
- (B) REGISTRATION NUMBER: 31,274
- (C) REFERENCE/DOCKET NUMBER: 4185.204-WO

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 212 867 0123
- (B) TELEFAX: 212 878 9655

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2418 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Polyporus pinsitus*

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 414..464

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 534..589

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 710..764

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 879..934

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1001..1050

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1147..1197

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1354..1410

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1609..1662

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: join (413..465, 533..590, 709..765, 878..935,
 1000..1051, 1146..1198, 1353..1411, 1608..1663)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGATTTCTGA CACCGGTGCA ATCTTGACAC TGTACCAACC GGGCAAGTCT CGTCCTTGGT	60
TCTCGGGGACT GGCGCCGGT CGCTACCCCT TGGTCATTCA CTCTACCAGA GCGCTGGCTT	120
CGCCGAGGTA TAAAGGATGT TGCGCGACAC CCTCAACACC CCAACTCAAG CCCCACTTGA	180
GCTTTTGCGA GATCCTCCAC ATACCACTCA CTACTTTCAA GTTCTTCAAC ATG TCG AGG	239
Met Ser Arg	
1	
TTT CAC TCT CTT CTC GCT TTC GTC GTT GCT TCC CTT ACG GCT GTG GCC	287
Phe His Ser Leu Leu Ala Phe Val Val Ala Ser Leu Thr Ala Val Ala	
5 10 15	
CAC GCT GGT ATC GGT CCC GTC GCC GAC CTA ACC ATC ACC AAC GCA GCG	335
His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr Ile Thr Asn Ala Ala	
20 25 30 35	
GTC AGC CCC GAC GGG TTT TCT CGC CAG GCC GTC GTC GTG AAC GGC GGC	383
Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val Asn Gly Gly	
35 40 45	
ACC CCT GGC CCT CTC ATC ACG GGT AAC ATG GTTCGTCTCG GCTCGCACTA	433
Thr Pro Gly Pro Leu Ile Thr Gly Asn Met	
50 55	
GGGGGTTGTGA TCGTTCCCTGA CGTTGTTGGA G GGG GAT CGC TTC CAG CTC AAT GTC ATC	491

	Gly Asp Arg Phe Gln Leu Asn Val Ile	
	60	65
GAC AAC CTT ACC AAC CAC ACG ATG GTG AAG AGC ACG AGT ATT GTGAGCTGCT Asp Asn Leu Thr Asn His Thr Met Val Lys Ser Thr Ser Ile 70 75		543
ATTTCTCCGG ACGGGGCTTC ATTGTGCTAA TAATCGTCGT GTGCAG CAC TGG CAC GGT His Trp His Gly 80		601
TTC TTC CAG AAG GGT ACC AAC TGG GCC GAC GGT CCC GCC TTC ATC AAC Phe Phe Gln Lys Gly Thr Asn Trp Ala Asp Gly Pro Ala Phe Ile Asn 85 90 95		649
CAG TGC CCG ATC TCA TCT GGT CAC TCG TTC CTG TAC GAC TTC CAG GTT Gln Cys Pro Ile Ser Ser Gly His Ser Phe Leu Tyr Asp Phe Gln Val 100 105 110 115		697
CCT GAC CAG GCT GTAAGTACGG TCGTTATGGA GTATACTGCG CATTGCTAAA Pro Asp Gln Ala		749
CCACATGGTG AACAG GGT ACC TTC TGG TAT CAC AGT CAC TTG TCT ACG CAG Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln 120 125 130		800
TAC TGT GAT GGT TTG AGG GGT CCG TTC GTT GTT TAC GAC CCG AAT GAC Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val Tyr Asp Pro Asn Asp 135 140 145		848
CCG GCC GCC GAC CTG TAC GAC GTC GAC AAC GTAAGGACGA ATTGAAACCG Pro Ala Ala Asp Leu Tyr Asp Val Asp Asn 150 155		898
TAAATACTTG CTTACTGATA CTTCTCGATG AATTAG GAC GAC ACT GTC ATT Asp Asp Thr Val Ile 160		949
ACC CTT GTG GAT TGG TAC CAC GTC GCC GCG AAG CTG GGC CCC GCA TTC Thr Leu Val Asp Trp Tyr His Val Ala Ala Lys Leu Gly Pro Ala Phe 165 170 175		997
CCT GTAAGTCCAT GAGTATTCTG CTGTTGAATC TGTCTTAAC GTGCATATCA CTC Pro Leu 180		1053
GGC GCC GAC ACC CTC ATC AAC GGT AAG GGA CGC TCC CCC AGC ACG Gly Ala Asp Ala Thr Leu Ile Asn Gly Lys Gly Arg Ser Pro Ser Thr 185 190 195		1101
ACC ACC GCG GAC CTC TCA GTT ATC AGC GTC ACC CCG GGT AAA CGC Thr Thr Ala Asp Leu Ser Val Ile Ser Val Thr Pro Gly Lys Arg 200 205 210		1146
GTATGCTATA TCTTATCTTA TCTGATGGCA TTTCTCTGAG ACATTCTCCA G		1197
TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC GAC CCC AAC TAC ACG TTC Tyr Arg Phe Arg Leu Val Ser Leu Ser Cys Asp Pro Asn Tyr Thr Phe 215 220 225		1245
AGC ATC GAT GGT CAC AAC ATG ACG ATC GAG ACC GAC TCA ATC AAC Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Thr Asp Ser Ile Asn 230 235 240		1293
ACG GCG CCC CTC GTC GTC GAC TCC ATT CAG ATC TTC GCC GCC CAG CGT Thr Ala Pro Leu Val Val Asp Ser Ile Gln Ile Phe Ala Ala Gln Arg 245 250 255		1341
TAC TCC TTC GTG GTAAGTTCGA TTTCATCCTCT AACGTTGGTC GCTGTTAGTG		1393

Tyr Ser Phe Val		
260		
ATCGTATGGT CATGTAG CTC GAG GCC AAC CAG GCC GTC GAC AAC TAC TGG		1443
Leu Glu Ala Asn Gln Ala Val Asp Asn Tyr Trp		
265 270		
ATT CGC GCC AAC CCG AAC TTC GGT AAC GTC GGG TTC ACC GGC GGC ATT		1491
Ile Arg Ala Asn Pro Asn Phe Gly Asn Val Gly Phe Thr Gly Ile		
275 280 285 290		
AAC TCG GCT ATC CTC CGC TAC GAT GGT GCC GCT GCC GTG GAG CCC ACC		1539
Asn Ser Ala Ile Leu Arg Tyr Asp Gly Ala Ala Ala Val Glu Pro Thr		
295 300 305		
ACA ACG CAA ACC ACG TCG ACT GCG CCG CTC AAC GAG GTC AAC CTG CAC		1587
Thr Thr Gln Thr Thr Ser Thr Ala Pro Leu Asn Glu Val Asn Leu His		
310 315 320		
CCG CTG GTT ACC ACC GCT GTG GTATGTAATA TTGTCGGTAA TGTAATACAT		1638
Pro Leu Val Thr Thr Ala Val		
325		
TGTTGCTGAC CTCGACCCCC ACAG CCT GGC TCG CCC GTC GCT GGT GGT GTC		1689
Pro Gly Ser Pro Val Ala Gly Gly Val		
330 335		
GAC CTG GCC ATC AAC ATG GCG TTC AAC TTC AAC GGC ACC AAC TTC TTC		1737
Asp Leu Ala Ile Asn Met Ala Phe Asn Phe Asn Gly Thr Asn Phe Phe		
340 345 350		
ATC AAC GGC ACG TCT TTC ACG CCC CCG ACC GTG CCT GTC CTG CTC CAG		1785
Ile Asn Gly Thr Ser Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln		
355 360 365 370		
ATC ATC AGC GGC GCG CAG AAC GCG CAG GAC CTC CTG CCC TCC GGT AGC		1833
Ile Ile Ser Gly Ala Gln Asn Ala Gln Asp Leu Leu Pro Ser Gly Ser		
375 380 385		
GTC TAC TCG CTT CCC TCG AAC GCC GAC ATC GAG ATC TCC TTC CCC GCC		1881
Val Tyr Ser Leu Pro Ser Asn Ala Asp Ile Glu Ile Ser Phe Pro Ala		
390 395 400		
ACC GCC GCC CCC GGT GCG CCC CAC CCC TTC CAC TTG CAC GGG CAC		1929
Thr Ala Ala Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His		
405 410 415		
GCG TTC GCG GTC CGC AGC GCC GGC AGC ACG GTT TAC AAC TAC GAC		1977
Ala Phe Ala Val Val Arg Ser Ala Gly Ser Thr Val Tyr Asn Tyr Asp		
420 425 430		
AAC CCC ATC TTC CGC GAC GTC GTC AGC ACG GGG ACG CCT GCG GCC GGT		2025
Asn Pro Ile Phe Arg Asp Val Val Ser Thr Gly Thr Pro Ala Ala Gly		
435 440 445 450		
GAC AAC GTC ACC ATC CGC TTC CGC ACC GAC AAC CCC GGC CCG TGG TTC		2073
Asp Asn Val Thr Ile Arg Phe Arg Thr Asp Asn Pro Gly Pro Trp Phe		
455 460 465		
CTC CAC TGC CAC ATC GAC TTC CAC CTC GAG GCC GGC TTC GCC GTC GTG		2121
Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Val Val		
470 475 480		
TTC GCG GAG GAC ATC CCC GAC GTC GCG TCG GCG AAC CCC GTC CCC CAG		2169
Phe Ala Glu Asp Ile Pro Asp Val Ala Ser Ala Asn Pro Val Pro Gln		
485 490 495		
GCG TGG TCC GAC CTC TGT CCG ACC TAC GAC GCG CTC GAC CCG AGC GAC		2217

Ala Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Pro Ser Asp	
500 505 510	
CAG TAAATGGCTT GCGCCGGTCG ATGATAGGAT ATGGACGGTG AGTTCGCACT	2270
Gln	
515	
TGCAATAACGG ACTCTCGCCT CATTATGGTT ACACACTCGC TCTGGATCTC TCGCCTGTCG	2330
ACAGAACAAA CTTGTATAAT TCGCTTAATG GTTGAAACAA ATGGAATATT GGGGTACTAT	2390
GCACGCATCT CGCTGGGTGA GCTTTCGT	2418

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 520 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Polyporus pinsitus*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Arg Phe His Ser Leu Leu Ala Phe Val Val Ala Ser Leu Thr	
1 5 10 15	
Ala Val Ala His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr Ile Thr	
20 25 30	
Asn Ala Ala Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val	
35 40 45	
Asn Gly Gly Thr Pro Gly Pro Leu Ile Thr Gly Asn Met Gly Asp Arg	
50 55 60	
Phe Gln Leu Asn Val Ile Asp Asn Leu Thr Asn His Thr Met Val Lys	
65 70 75 80	
Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn Trp	
85 90 95	
Ala Asp Gly Pro Ala Phe Ile Asn Gln Cys Pro Ile Ser Ser Gly His	
100 105 110	
Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly Thr Phe Trp	
115 120 125	
Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro	
130 135 140	
Phe Val Val Tyr Asp Pro Asn Asp Pro Ala Ala Asp Leu Tyr Asp Val	
145 150 155 160	
Asp Asn Asp Asp Thr Val Ile Thr Leu Val Asp Trp Tyr His Val Ala	
165 170 175	
Ala Lys Leu Gly Pro Ala Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile	
180 185 190	
Asn Gly Lys Gly Arg Ser Pro Ser Thr Thr Ala Asp Leu Ser Val	
195 200 205	
Ile Ser Val Thr Pro Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Leu	

210

215

220

Ser Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Met Thr
 225 230 235 240
 Ile Ile Glu Thr Asp Ser Ile Asn Thr Ala Pro Leu Val Val Asp Ser
 245 250 255
 Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val Leu Glu Ala Asn
 260 265 270
 Gln Ala Val Asp Asn Tyr Trp Ile Arg Ala Asn Pro Asn Phe Gly Asn
 275 280 285
 Val Gly Phe Thr Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Asp Gly
 290 295 300
 Ala Ala Ala Val Glu Pro Thr Thr Gln Thr Thr Ser Thr Ala Pro
 305 310 315 320
 Leu Asn Glu Val Asn Leu His Pro Leu Val Thr Thr Ala Val Pro Gly
 325 330 335
 Ser Pro Val Ala Gly Gly Val Asp Leu Ala Ile Asn Met Ala Phe Asn
 340 345 350
 Phe Asn Gly Thr Asn Phe Phe Ile Asn Gly Thr Ser Phe Thr Pro Pro
 355 360 365
 Thr Val Pro Val Leu Leu Gln Ile Ile Ser Gly Ala Gln Asn Ala Gln
 370 375 380
 Asp Leu Leu Pro Ser Gly Ser Val Tyr Ser Leu Pro Ser Asn Ala Asp
 385 390 395 400
 Ile Glu Ile Ser Phe Pro Ala Thr Ala Ala Ala Pro Gly Ala Pro His
 405 410 415
 Pro Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly
 420 425 430
 Ser Thr Val Tyr Asn Tyr Asp Asn Pro Ile Phe Arg Asp Val Val Ser
 435 440 445
 Thr Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Arg Thr
 450 455 460
 Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu
 465 470 475 480
 Glu Ala Gly Phe Ala Val Val Phe Ala Glu Asp Ile Pro Asp Val Ala
 485 490 495
 Ser Ala Asn Pro Val Pro Gln Ala Trp Ser Asp Leu Cys Pro Thr Tyr
 500 505 510
 Asp Ala Leu Asp Pro Ser Asp Gln
 515 520

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2880 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ix) FEATURE:
 - (A) NAME/KEY: intron

(B) LOCATION: 544..592

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 837..899

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1014..1066

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1133..1187

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1284..1342

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1752..1815

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1873..1928

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 2136..2195

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: join(364..543, 593..661, 716..835, 900..1013,
 1067..1132, 1188..1283, 1343..1498, 1554..1751,
 1816..1872, 1929..2135, 2196..2489)

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 662..715

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION: 1499..1553

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GC GGCGCACA AACCGTGGGA	GCC AACACAC	TCCC GTCCAC	TCTCACACTG	GCCAGATTG	60
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TG CCTGCCG	ATTCAATTGT	GCGCCAGTCG	GGCATCCGGA	TGGCTCTACC	180
ACTGGAAGAG	AACACCGAGG	TCATGCATTC	TGGCCAAGTG	CGGCCAAAGG	240
GGT GCGGATA	CTTAAAGGGC	GGCGCGGGGA	GGCCTGTCTA	CCAAGCTCAA	300
GGTTCCCAGT	CTCCGCCACC	CTCCTCTTCC	CCCACACAGT	CGCTCCATAG	360
GCC	ATG GGT CTG CAG CGA	TTC AGC TTC	GTC ACC CTC	GCG CTC GTC	408
Met	Gly Leu Gln Arg	Phe Ser Phe	Phe Val Thr	Leu Ala Leu Val	
1	5	10	15		
GCT CGC TCT CTT	GCA GCC ATC GGG CCG	GTG GCG AGC	CTC GTC GTC	GCG	456
Ala Arg Ser Leu	Ala Ala Ile	Gly Pro Val	Ala Ser Leu Val	Val Ala	
20	25	30			
AAC GCC CCC GTC TCG CCC GAC GGC	TTC CTT CGG GAT	GCC ATC GTG GTC			504
Asn Ala Pro Val Ser Pro Asp	Gly Phe Leu Arg Asp	Ala Ile Val Val			
35	40	45			

AAC GGC GTG GTC CCT TCC CCG CTC ATC ACC GGG AAG AAG GTCGGCGTGT Asn Gly Val Val Pro Ser Pro Leu Ile Thr Gly Lys Lys 50 55 60	553
TCGTCGTCGT CCTACTCCTT TGCTGACAGC GATCTACAG GGA GAC CGC TTC CAG Gly Asp Arg Phe Gln 65	607
CTC AAC GTC GTC GAC ACC TTG ACC AAC CAC AGC ATG CTC AAG TCC ACT Leu Asn Val Val Asp Thr Leu Thr Asn His Ser Met Leu Lys Ser Thr 70 75 80	655
AGT ATC GTAAAGTGTGA CGATCCGAAT GTGACATCAA TCGGGGCTAA TTAACCGCC Ser Ile	711
ACAG CAC TGG CAC GGC TTC TTC CAG GCA GGC ACC AAC TGG GCA GAA GGA His Trp His Gly Phe Phe Gln Ala Gly Thr Asn Trp Ala Glu Gly 85 90 95	760
CCC GCG TTC GTC AAC CAG TGC CCT ATT GCT TCC GGG CAT TCA TTC CTG Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser Phe Leu 100 105 110	808
TAC GAC TTC CAT GTG CCC GAC CAG GCA GTAAAGCAGGA TTTTCTGGGG Tyr Asp Phe His Val Pro Asp Gln Ala 115 120	855
TCCCCGTGTG ATGCAATGTT CTCATGCTCC GACGTGATCG ACAG GGG ACG TTC TGG Gly Thr Phe Trp 125	911
TAC CAC AGT CAT CTG TCT ACG CAG TAC TGT GAC GGG CTG CGG GGG CCG Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro 130 135 140	959
TTC GTC GTG TAC GAC CCC AAG GAC CCG CAC GCC AGC CGT TAC GAT GTT Phe Val Val Tyr Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val 145 150 155	1007
GAC AAT GTACGTGCGC CACGGAGTAT ATCACACAGC ATGCGTTGAC GTCGGGCCAA Asp Asn 160	1063
CAG GAG AGC ACG GTC ATC ACG TTG ACC GAC TGG TAC CAC ACC GCT GCC Glu Ser Thr Val Ile Thr Leu Thr Asp Trp Tyr His Thr Ala Ala 165 170 175	1111
CGG CTC GGT CCC AAG TTC CCA GTAAAGCTCGC AATGGCTTAG TGTTCACAGG Arg Leu Gly Pro Lys Phe Pro 180	1162
TTCTTTGCTT ATGTTGCTTC GATAG CTC GGC GCG GAC GCC ACG CTC ATC AAC Leu Gly Ala Asp Ala Thr Leu Ile Asn 185 190	1214
GGT CTG GGG CGG TCG GCC TCG ACT CCC ACC GCT GCG CTT GCC GTG ATC Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala Val Ile 195 200 205	1262
AAC GTC CAG CAC GGA AAG CGC GTGAGCATTC TCTTGTATGC CATTCAATG Asn Val Gln His Gly Lys Arg 210 215	1313
CTTTGTGCTG ACCTATCGGA ACCGGCGAG TAC CGC TTC CGT CTC GTT TCG ATC Tyr Arg Phe Arg Leu Val Ser Ile 220	1366

TCG TGT GAC CCG AAC TAC ACG TTC AGC ATC GAC GGG CAC AAC CTG ACC Ser Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr 225 230 235	1414
GTC ATC GAG GTC GAC GGC ATC AAT AGC CAG CCT CTC CTT GTC GAC TCT Val Ile Glu Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser 240 245 250 255	1462
ATC CAG ATC TTC GCC GCA CAG CGC TAC TCC TTC GTG GTAAAGTCCTG Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val 260 265	1508
GCTTGTCGAT GCTCCAAAGT GGCCCTCACTC ATATACTTTG GTTAG TTG AAT GCG Leu Asn Ala 270	1562
AAT CAA ACG GTG GGC AAC TAC TGG GTT CGT GCG AAC CCG AAC TTC GGA Asn Gln Thr Val Gly Asn Tyr Trp Val Arg Ala Asn Pro Asn Phe Gly 275 280 285	1610
ACG GTT GGG TTC GCC GGG GGG ATC AAC TCC GCC ATC TTG CGC TAC CAG Thr Val Gly Phe Ala Gly Ile Asn Ser Ala Ile Leu Arg Tyr Gln 290 295 300	1658
GGC GCA CCG GTC GCC GAG CCT ACC ACG ACC CAG ACG CCG TCG GTG ATC Gly Ala Pro Val Ala Glu Pro Thr Thr Gln Thr Pro Ser Val Ile 305 310 315	1706
CCG CTC ATC GAG ACG AAC TTG CAC CCG CTC GCG CGC ATG CCA GTG Pro Leu Ile Glu Thr Asn Leu His Pro Leu Ala Arg Met Pro Val 320 325 330	1751
GTATGTCCTCT TTTTCTGATC ATCTGAGTTG CCCGTTGTTG ACCGCATTAT GTGTTACTAT	1811
CTAG CCT GGC AGC CCG ACA CCC GGG GGC GTC GAC AAG GCG CTC AAC CTC Pro Gly Ser Pro Thr Pro Gly Val Asp Lys Ala Leu Asn Leu 335 340 345	1860
GCG TTT AAC TTC GTAAAGTATCT CTACTACTTA GGCTGGAGGC TCGTCGCTGA Ala Phe Asn Phe 350	1912
TCATACGGTG CTTCAAG AAC GGC ACC AAC TTC TTC ATC AAC AAC GCG ACT Asn Gly Thr Asn Phe Phe Ile Asn Asn Ala Thr 355 360	1961
TTC ACG CCG CCG ACC GTC CCG GTC CTC CTC CAG ATT CTG AGC GGT GCG Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala 365 370 375	2009
CAG ACC GCA CAA GAC CTG CTC CCC GCA GGC TCT GTC TAC CCG CTC CCG Gln Thr Ala Gln Asp Leu Leu Pro Ala Gly Ser Val Tyr Pro Leu Pro 380 385 390 395	2057
GCC CAC TCC ACC ATC GAG ATC ACG CTG CCC GCG ACC GCC TTG GCC CCG Ala His Ser Thr Ile Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro 400 405 410	2105
GGT GCA CCG CAC CCC TTC CAC CTG CAC GGT GTATGTTCCC CTGCCTTCCC Gly Ala Pro His Pro Phe His Leu His Gly 415 420	2155
TTCTTATCCC CGAACCGAGTG CTCACGTCCG TCCCATCTAG CAC GCC TTC GCG GTC His Ala Phe Ala Val 425	2210
GTT CGC AGC GCG GGG AGC ACC ACG TAT AAC TAC AAC GAC CCG ATC TTC Val Arg Ser Ala Gly Ser Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe 430 435 440	2258

CGC GAC GTC GTG AGC ACG GGC ACG CCC GCC GCG GGC GAC AAC GTC ACG Arg Asp Val Val Ser Thr Gly Thr Pro Ala Ala Gly Asp Asn Val Thr 445 450 455	2306
ATC CGC TTC CAG ACG GAC AAC CCC GGG CCG TGG TTC CTC CAC TGC CAC Ile Arg Phe Gln Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His 460 465 470	2354
ATC GAC TTC CAC CTC GAC GCA GGC TTC GCG ATC GTG TTC GCA GAG GAC Ile Asp Phe His Leu Asp Ala Gly Phe Ala Ile Val Phe Ala Glu Asp 475 480 485 490	2402
GTT GCG GAC GTG AAG GCG GCG AAC CCG GTT CCG AAG GCG TGG TCG GAC Val Ala Asp Val Lys Ala Ala Asn Pro Val Pro Lys Ala Trp Ser Asp 495 500 505	2450
CTG TGC CCG ATC TAC GAC GGG CTG AGC GAG GCT AAC CAG TGAGCGGAGG Leu Cys Pro Ile Tyr Asp Gly Leu Ser Glu Ala Asn Gln 510 515	2499
CGCTGGTGT GAGCGTAAAG CTCGGCGTC GACCTGGGG GTTGAAGGTG TTCTGATTGA	2559
AATGGTCTTT GGGTTTATTT GTTGTATTTC TAACTCGTT CTCTACGCAA GGACCGAGGA	2619
TTGTATAGGA TGAAGTAAC TCCCTAATGT ATTATGATAT CAATTGACGG AGGCATGGAC	2679
TGCGAAGTGT GTACAATGTG GTAGTGGTCT AGGCCTTGGA GACAAGCTGT GGATTTTCT	2739
TGGGGGATGA AGAGGCCTGA AGGCTGAGAG CTATGCTATG CCTAGTGACG TGGTTATAGT	2799
AAATGTCCAT TACATTGACC AAGAACGACA AGAACCATAA GCTTGCTGAG GATAGATGGG	2859
GGCGCGTCCG CGAACGACTT G	2880

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 519 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu Val Ala 1 5 10 15
Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala Asn 20 25 30
Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val Asn 35 40 45
Gly Val Val Pro Ser Pro Leu Ile Thr Gly Lys Lys Gly Asp Arg Phe 50 55 60
Gln Leu Asn Val Val Asp Thr Leu Thr Asn His Ser Met Leu Lys Ser 65 70 75 80
Thr Ser Ile His Trp His Gly Phe Phe Gln Ala Gly Thr Asn Trp Ala 85 90 95
Glu Gly Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser 100 105 110
Phe Leu Tyr Asp Phe His Val Pro Asp Gln Ala Gly Thr Phe Trp Tyr 115 120 125

His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe
 130 135 140
 Val Val Tyr Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val Asp
 145 150 155 160
 Asn Glu Ser Thr Val Ile Thr Leu Thr Asp Trp Tyr His Thr Ala Ala
 165 170 175
 Arg Leu Gly Pro Lys Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile Asn
 180 185 190
 Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala Val Ile
 195 200 205
 Asn Val Gln His Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Ile Ser
 210 215 220
 Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr Val
 225 230 235 240
 Ile Glu Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser Ile
 245 250 255
 Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val Leu Asn Ala Asn Gln
 260 265 270
 Thr Val Gly Asn Tyr Trp Val Arg Ala Asn Pro Asn Phe Gly Thr Val
 275 280 285
 Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Gln Gly Ala
 290 295 300
 Pro Val Ala Glu Pro Thr Thr Gln Thr Pro Ser Val Ile Pro Leu
 305 310 315 320
 Ile Glu Thr Asn Leu His Pro Leu Ala Arg Met Pro Val Pro Gly Ser
 325 330 335
 Pro Thr Pro Gly Val Asp Lys Ala Leu Asn Leu Ala Phe Asn Phe
 340 345 350
 Asn Gly Thr Asn Phe Phe Ile Asn Asn Ala Thr Phe Thr Pro Pro Thr
 355 360 365
 Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Gln Thr Ala Gln Asp
 370 375 380
 Leu Leu Pro Ala Gly Ser Val Tyr Pro Leu Pro Ala His Ser Thr Ile
 385 390 395 400
 Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro Gly Ala Pro His Pro
 405 410 415
 Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly Ser
 420 425 430
 Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe Arg Asp Val Val Ser Thr
 435 440 445
 Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Gln Thr Asp
 450 455 460
 Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Asp
 465 470 475 480
 Ala Gly Phe Ala Ile Val Phe Ala Glu Asp Val Ala Asp Val Lys Ala
 485 490 495

Ala Asn Pro Val Pro Lys Ala Trp Ser Asp Leu Cys Pro Ile Tyr Asp
500 505 510

Gly Leu Ser Glu Ala Asn Gln
515

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3102 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Polyporus pinsitus*

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 666..720

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 790..845

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1125..1182

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1390..1450

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1607..1661

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1863..1918

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1976..2025

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 2227..2285

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 2403..2458

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 2576..2627

(ix) FEATURE:

- (A) NAME/KEY: CDS

- (B) LOCATION: join (665..721, 789..846, 1124..1183, 1389..1451, 1606..1662, 1862..1919, 1975..2026, 2226..2286, 2402..2459, 2575..2628).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTTCCGACT AAACCAATCT CAGNCCGCTT CCTCCTAGGG AACCGAGCGA TGTGGCGGCC

60

CTCTCTATCC AAGCTGTCCA TAAGAAGACG TTCAAATGCC GCAGCAAGCG AGGAAATAAG	120
CATCTAACAG TGTGTTTCCC ATAGTCGCAT TTGCGCCGCC TGTGGACCG ACGCCCTAG	180
AGCGCTTGG GAAACGTCGC AAGTGGCGGG TGTATTACGT GTAGACGAGA CGGTATTTGT	240
CTCATCATTC CCGTGCTTCA GGTTGACACA GCCCAAAGGT CTATGTACGG CCCTTCACAT	300
TCCCTGACAC ATTGACGCAA CCCTCGGTGC GCCTCCGACA GTGCCCTCGGT TGTAGTATCG	360
GGACGCCCTA GGATGCAAGA TTGGAAGTCA CCAAGGCCCG AAGGGTATAA AATACCGAGA	420
GGTCCTACCA CTTCTGCATC TCCAGTCGCA GAGTTCCCTCT CCCCTGCCAG CCACAGCTCG	480
AG ATG TCC TTC TCT AGC CTT CGC CGT GCC TTG GTC TTC CTG GGT GCT	527
Met Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala	
1 5 10 15	
TGC AGC AGT GCG CTG GCC TCC ATC GGC CCA GTC ACT GAG CTC GAC ATC	575
Cys Ser Ser Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile	
20 25 30	
GTT AAC AAG GTC ATC GCC CCG GAT GGC GTC GCT CGT GAT ACA GTC CTC	623
Val Asn Lys Val Ile Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu	
35 40 45	
GCC GGG GGC ACG TTC CCG GGC CCA CTC ATC ACA GGA AAG AAG	665
Ala Gly Gly Thr Phe Pro Gly Pro Leu Ile Thr Gly Lys Lys	
50 55 60	
GTATGCTAAG TAGTCCCGCC CCCATCATCC TGTGGCTGAC GTTCGACGCC GCCAG	720
GGT GAC AAC TTC CGC ATC AAC GTC GTC GAC AAG TTG GTT AAC CAG ACT	768
Gly Asp Asn Phe Arg Ile Asn Val Val Asp Lys Leu Val Asn Gln Thr	
65 70 75	
ATG CTG ACA TCC ACC ACC ATT GTATGTCACT AGCTCTCGCT ATCTCGAGAC	819
Met Leu Thr Ser Thr Thr Ile	
80	
CCGCTGACCG ACAACATTTG CCGTAG CAC TGG CAC GGG ATG TTC CAG CAT	859
His Trp His Gly Met Phe Gln His	
85 90	
ACG ACG AAC TGG GCG GAT GGT CCC GCC TTT GTG ACT CAA TGC CCT ATC	917
Thr Thr Asn Trp Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile	
95 100 105	
ACC ACT GGT GAT TTC CTG TAC AAC TTC CGC GTG CCC GAC CAG ACA	965
Thr Thr Gly Asp Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr	
110 115 120	
GTACGCAAAG GGCAGCATGC GTACTCAAAG ACATCTCTAA GCATTTGCTA CCTAG	1020
GGA ACG TAC TGG TAC CAT AGC CAT CTG GCC TTG CAG TAC TGT GAT GGG	1068
Gly Thr Tyr Trp Tyr His Ser His Leu Ala Leu Gln Tyr Cys Asp Gly	
125 130 135 140	
CTT CGC GGC CCC CTG GTG ATT TAC GAT CCC CAT GAT CCG CAG GCA TAC	1116
Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro His Asp Pro Gln Ala Tyr	
145 150 155	
CTG TAT GAC GTC GAT GAC GTACGCAGCA CAGTTCCCT AAAACGGTTA	1164
Leu Tyr Asp Val Asp Asp	
160	
ACTTCTAATT CTGTAAATAT CTTCATAG GAG AGC ACC GTT ATC ACT CTG	1213
Glu Ser Thr Val Ile Thr Leu	
165	

GCA GAC TGG TAC CAT ACC CCG GCG CCT CTG CTG CCG CCT GCC GCG Ala Asp Trp Tyr His Thr Pro Ala Pro Leu Leu Pro Pro Ala Ala 170 175 180	1258
GTACGCCCTCC ACACATCTGC ACAGCGTTCC GTATCTCATA CCCTTAAAGT TTATCGGACA	1318
ACT TTG ATT AAT GGC CTG GGT CGC TGG CCT GGC AAC CCC ACC GCC GAC Thr Leu Ile Asn Gly Leu Gly Arg Trp Pro Gly Asn Pro Thr Ala Asp 185 190 195 200	1366
CTA GCC GTC ATC GAA GTC CAG CAC GGA AAG CGC GTATGTCATA GCTCGGTTAT Leu Ala Val Ile Glu Val Gln His Gly Lys Arg 205 210	1419
CTATTCATAC TCGCGGCCTC GAAGCTAAAA CCTTGTTCGA G TAC CGG TTC CGA Tyr Arg Phe Arg 215	1472
CTG GTC AGC ACC TCA TGC GAC CCC AAC TAC AAC TTC ACT ATC GAT GGC Leu Val Ser Thr Ser Cys Asp Pro Asn Tyr Asn Phe Thr Ile Asp Gly 220 225 230	1520
CAC ACC ATG ACA ATC ATC GAG GCG GAT GGG CAG AAC ACC CAG CCA CAC His Thr Met Thr Ile Ile Glu Ala Asp Gly Gln Asn Thr Gln Pro His 235 240 245	1568
CAA GTC GAC GGA CTT CAG ATC TTC GCG GCA CAG CGG TAC TCC TTC GTT Gln Val Asp Gly Leu Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val 250 255 260	1616
GTATGTTTTC CGCATTTCGG GAAAAGGAAT TGCGCTGACA GCTCGAGTGT GCGTAG	1672
CTT AAC GCT AAC CAA GCG GTC AAC AAC TAC TGG ATC CGT GCG AAC CCT Leu Asn Ala Asn Gln Ala Val Asn Asn Tyr Trp Ile Arg Ala Asn Pro 265 270 275	1720
AAC CGT GCT AAC ACT ACG GGC TTC GCC AAC GGC ATC AAC TCC GCC ATC Asn Arg Ala Asn Thr Thr Gly Phe Ala Asn Gly Ile Asn Ser Ala Ile 280 285 290 295	1768
CTG CGC TAC AAG GGG GCG CCG ATT AAG GAG CCT ACG ACG AAC CAG ACT Leu Arg Tyr Lys Gly Ala Pro Ile Lys Glu Pro Thr Thr Asn Gln Thr 300 305 310	1816
ACC ATC CGG AAC TTT TTG TGG GAG ACG GAC TTG CAC CCG CTC ACT GAC Thr Ile Arg Asn Phe Leu Trp Glu Thr Asp Leu His Pro Leu Thr Asp 315 320 325	1864
CCA CGT GCA GTAAAGTTCTA CACAGTCACC AACGGTGAGC TGTTGTCTGA Pro Arg Ala 330	1913
TTGCACTGTG TTATAG CCT GGC CTT CCT TTC AAG GGG GGC GTT GAC CAC Pro Gly Leu Pro Phe Lys Gly Gly Val Asp His 335 340	1962
GCT TTG AAC CTC AAC CTC ACT TTC GTACGTAGCG CCTCAGATAT CGAGTAGTCT	2016
Ala Leu Asn Leu Asn Leu Thr Phe 345	
ATCTCCTGAC CGATTGACAG AAT GGA TCG GAG TTC TIC ATC AAC GAT GCG Asn Gly Ser Glu Phe Ile Asn Asp Ala 350 355	2066
CCT TTC GTC CCT CCG ACT GTC CCG GTG CTA CTG CAG ATC CTG AAC GGA Pro Phe Val Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Asn Gly 360 365 370 375	2114

ACG CTC GAC GCG AAC GAC CTC CTG CCG CCC GGC AGC GTC TAC AAC CTT Thr Leu Asp Ala Asn Asp Leu Leu Pro Pro Gly Ser Val Tyr Asn Leu 380 385 390	2162
CCT CCG GAC TCC ACC ATC GAG CTG TCC ATT CCC GGA GGT GTG ACG GGT Pro Pro Asp Ser Thr Ile Glu Leu Ser Ile Pro Gly Gly Val Thr Gly 395 400 405	2210
GGC CCG CAC CCA TTC CAT TTG CAC GGG GTAATAATCT CTCTTTATAC Gly Pro His Pro Phe His Leu His Gly 410 415	2257
TTTGGTCTCC CGATGCTGAC TTTCACTGCT CATCTTCAG CAC GCT TTC TCC GTC His Ala Phe Ser Val 420	2311
GTG CGT AGC GCC GGC AGC ACC GAA TAC AAC TAC GCG AAC CCG GTG AAG Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr Ala Asn Pro Val Lys 425 430 435	2359
CGC GAC ACG GTC AGC ATT GGT CTT GCG GGC GAC AAC GTC ACC GTG CGC Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp Asn Val Thr Val Arg 440 445 450	2407
TTC GTG GTATGTTTA CAGCCTCTCT ATCTCCGTGG GCGTTGGAA GTTGACTGGG Phe Val 455	2463
GCGTAG ACC GAC AAC CCC GGC CCG TGG TTC CTC CAC TGT CAC ATC GAC Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp 460 465	2511
TTC CAT TTG CAA GCA GGC CTC GCC ATC GTG TTC GCG GAG GAC GCG CAG Phe His Leu Gln Ala Gly Leu Ala Ile Val Phe Ala Glu Asp Ala Gln 470 475 480 485	2559
GAC ACG AAG CTT GTG AAC CCC GTC CCT GTACGTCTTC TGGATGCCATG Asp Thr Lys Leu Val Asn Pro Val Pro 490	2606
CGCTCCGCAC AGTGACTCAT CTTTGCAAC AG GAG GAC TGG AAC AAG CTG TGC Glu Asp Trp Asn Lys Leu Cys 495 500	2659
CCC ACC TTC GAT AAG GCG ATG AAC ATC ACG GTT TGAGCGATGC Pro Thr Phe Asp Lys Ala Met Asn Ile Thr Val 505 510	2702
GTGGCGCTCA TGGTCATTTT CTTGGAATCT TTGCATAGGG CTGCAGCACG CTGGATACTC	2762
TTCCTCTTAG CAGGATATTA TTAAATGACC CCTGCCTTA GTGCTTAGTT AGCTTTACTA	2822
CTGGTTGTAATGTACGC ATGCGTAATT CGGATAATGC TATCAATGTG TATATTATGA	2882
CACCGTCAT GCGCGATGCT TGAGTTGCAA GGTCGGTTTC CGATGCTCGA CATAAACGTT	2942
TCACTTACAT ACACATTGGG TCTAGAACTG GATCTATCCA TGTATACAAA AACTCCTCAT	3002
ACAGCTGACT GGGCGCTCT AGAGCATGGG TCCGATTGAT CAGATGTCGC GAACACGAGC	3062
CTCCTGAGCT CGAGGACTCT GAGAAGCGGC GGTGGCTTCT	3102

(2) INFORMATION FOR SEQ ID NO: 6

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 512 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Polyporus pinsitus*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala Cys
1 5 10 15

Ser Ser Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile Val
20 25 30

Asn Lys Val Ile Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu Ala
35 40 45

Gly Gly Thr Phe Pro Gly Pro Leu Ile Thr Gly Lys Lys Gly Asp Asn
50 55 60

Phe Arg Ile Asn Val Val Asp Lys Leu Val Asn Gln Thr Met Leu Thr
65 70 75 80

Ser Thr Thr Ile His Trp His Gly Met Phe Gln His Thr Thr Asn Trp
85 90 95

Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Thr Thr Gly Asp
100 105 110

Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr Gly Thr Tyr Trp
115 120 125

Tyr His Ser His Leu Ala Leu Gln Tyr Cys Asp Gly Leu Arg Gly Pro
130 135 140

Leu Val Ile Tyr Asp Pro His Asp Pro Gln Ala Tyr Leu Tyr Asp Val
145 150 155 160

Asp Asp Glu Ser Thr Val Ile Thr Leu Ala Asp Trp Tyr His Thr Pro
165 170 175

Ala Pro Leu Leu Pro Pro Ala Ala Thr Leu Ile Asn Gly Leu Gly Arg
180 185 190

Trp Pro Gly Asn Pro Thr Ala Asp Leu Ala Val Ile Glu Val Gln His
195 200 205

Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Thr Ser Cys Asp Pro Asn
210 215 220

Tyr Asn Phe Thr Ile Asp Gly His Thr Met Thr Ile Ile Glu Ala Asp
225 230 235 240

Gly Gln Asn Thr Gln Pro His Gln Val Asp Gly Leu Gln Ile Phe Ala
245 250 255

Ala Gln Arg Tyr Ser Phe Val Leu Asn Ala Asn Gln Ala Val Asn Asn
260 265 270

Tyr Trp Ile Arg Ala Asn Pro Asn Arg Ala Asn Thr Thr Gly Phe Ala
275 280 285

Asn Gly Ile Asn Ser Ala Ile Leu Arg Tyr Lys Gly Ala Pro Ile Lys
290 295 300

Glu Pro Thr Thr Asn Gln Thr Thr Ile Arg Asn Phe Leu Trp Glu Thr
305 310 315 320

Asp Leu His Pro Leu Thr Asp Pro Arg Ala Pro Gly Leu Pro Phe Lys
 325 330 335
 Gly Gly Val Asp His Ala Leu Asn Leu Asn Leu Thr Phe Asn Gly Ser
 340 345 350
 Glu Phe Phe Ile Asn Asp Ala Pro Phe Val Pro Pro Thr Val Pro Val
 355 360 365
 Leu Leu Gln Ile Leu Asn Gly Thr Leu Asp Ala Asn Asp Leu Leu Pro
 370 375 380
 Pro Gly Ser Val Tyr Asn Leu Pro Pro Asp Ser Thr Ile Glu Leu Ser
 385 390 395 400
 Ile Pro Gly Gly Val Thr Gly Gly Pro His Pro Phe His Leu His Gly
 405 410 415
 His Ala Phe Ser Val Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr
 420 425 430
 Ala Asn Pro Val Lys Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp
 435 440 445
 Asn Val Thr Val Arg Phe Val Thr Asp Asn Pro Gly Pro Trp Phe Leu
 450 455 460
 His Cys His Ile Asp Phe His Leu Gln Ala Gly Leu Ala Ile Val Phe
 465 470 475 480
 Ala Glu Asp Ala Gln Asp Thr Lys Leu Val Asn Pro Val Pro Glu Asp
 485 490 495
 Trp Asn Lys Leu Cys Pro Thr Phe Asp Lys Ala Met Asn Ile Thr Val
 500 505 510

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2860 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 851..905
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 1266..1320
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 1351..1376
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 1416..1468
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 1625..1683
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 1882..1934

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2202..2252

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2370..2425

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2543..2599

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: join(540..725, 782..850, 906..1025, 1086..1265,
1321..1350, 1377..1415, 1469..1624, 1684..1881,
1935..2201, 2253..2369, 2426..2542, 2600..2653)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGGGGCGCG TCAATGGTCC GTTTGCGAAC ACATATGCAG GATAAACAGT GCGAAATATC	60
AATGTGGCGG CGACACACAACC TCGCCGGCG ACACTCGACG CTGTTGATCA TGATCATGTC	120
TTGTGAGCAT TCTATACGCA GCCTTGGAAA TCTCAGGCGA ATTTGTCTGA ATTGCGCTGG	180
GAGGCTGGCA CGCCAGATCG GTGTGTCGGT GCAGTAGCCG ACGCAGCACC TGGCGGAAGC	240
CGACATCTCG GGTACGACTT GATCTCCGCC AGATCACTGC GGTTCCGCCA TCGGCCGGG	300
GGCCCATTCT GTGTGTGCGC TGTAGCACTC TGCATTCAAGG CTCAACGTAT CCATGCTAGA	360
GGACCGTCCA GCTGTTGGCG CACGATTGCG GCAGAAAAGCT GTACAGGCAG ATATAAGGAT	420
GTCCGTCCGT CAGAGACTCG TCACTCACAA GCCTCTTTCT CTCTTCGCCCT TTCCAGCCTC	480
TTCCAACGCC TGCCATCGTC CTCTTAGTTC GCTCGCCAT TCTTTCTGCG TAGTTAAC	539
ATG GGC AGG TTC TCA TCT CTC TGC GCG CTC ACC GCC GTC ATC CAC TCT Met Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile His Ser	587
1 5 10 15	
TTT GGT CGT GTC TCC GCC GCT ATC GGG CCT GTG ACC GAC CTC ACC ATC Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile	635
20 25 30	
TCC AAT GGG GAC GTT TCT CCC GAC GGC TTC ACT CGT GCC GCA GTG CTT Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu	683
35 40 45	
GCA AAC GGC GTC TTC CCG GGT CCT CTT ATC ACG GGA AAC AAG Ala Asn Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys	725
50 55 60	
GTACGTGGCA TGGCTTCAGT CTACACCCCTA CAAGCCTTCT AACTCTTTA CCACAG	781
GGC GAC AAC TTC CAG ATC AAT GTT ATC GAC AAC CTC TCT AAC GAG ACG Gly Asp Asn Phe Gln Ile Asn Val Ile Asp Asn Leu Ser Asn Glu Thr	829
65 70 75	
ATG TTG AAG TCG ACC TCC ATC GTATGTGCTT CTACTGCTTC TTAGTCTTGG	880
Met Leu Lys Ser Thr Ser Ile	
80 85	
CAATGGCTCA AGGTCTCCTC CGCAG CAT TGG CAC GGC TTC TTC CAG AAG GGT His Trp His Gly Phe Phe Gln Lys Gly	932
90	
ACT AAC TGG GCT GAT GGA GCT GCC TTC GTC AAC CAG TGC CCT ATC GCG	980

Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala		
95 100 105 110		
ACG GGG AAC TCT TTC CTT TAC GAC TTC ACC GCG ACG GAC CAA GCA		1025
Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala		
115 120 125		
GTCAGTGCCT GTGGCGCTTA TGTTTCCCG TAATCAGCAG CTAACACTCC GCACCCACAG		1085
Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly		
130 135 140		
TTG CGG GGC CCG ATG GTC GTA TAC GAC CCG AGT GAC CCG CAT GCG GAC		1181
Leu Arg Gly Pro Met Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp		
145 150 155		
CTT TAC GAC GTC GAC GAC GAG ACC ACG ATC ATC ACG CTC TCT GAT TGG		1229
Leu Tyr Asp Val Asp Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp		
160 165 170		
TAT CAC ACC GCT GCT TCG CTC GGT GCT GCC TTC CCG GTAAGTTAC		1275
Tyr His Thr Ala Ala Ser Leu Gly Ala Ala Phe Pro		
175 180 185		
CCCAGCCAC GGAGTTAAGA CCGGATCTAA CTGTAATACG TTTCAG ATT GGC TCG		1329
Ile Gly Ser		
GAC TCT ACC CTG ATT AAC GGC GTTGGCCGCT TCGCGGGTGG TGACAG ACT GAC		1382
Asp Ser Thr Leu Ile Asn Gly		
190 195		
CTT GCG GTT ATC ACT GTC GAG CAG GGC AAG CGC GTTAGTGATA CCCTCTACAG		1435
Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg		
200 205		
TTGACACTGT GCCATTGCTG ACAGTACTCT CAG TAC CGT ATG CGT CTT CTC TCG		1489
Tyr Arg Met Arg Leu Leu Ser		
210 215		
CTG TCT TGC GAC CCC AAC TAT GTC TTC TCC ATT GAC GGC CAC AAC ATG		1537
Leu Ser Cys Asp Pro Asn Tyr Val Phe Ser Ile Asp Gly His Asn Met		
220 225 230		
ACC ATC ATC GAG GCC GAC CAC AAC CAC GAG CCC CTC ACG GTT GAC		1585
Thr Ile Ile Glu Ala Asp Ala Val Asn His Glu Pro Leu Thr Val Asp		
235 240 245		
TCC ATC CAG ATC TAC GCC GGC CAA CGT TAC TCC TTC GTC GTACGTATTG		1634
Ser Ile Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Phe Val		
250 255 260		
CGAACAGCCA TGATCACGCC AAGCCCGATG CTAACCGGCC TACCCCTCAG CTT ACC		1689
Leu Thr		
GCT GAC CAG GAC ATC GAC AAC TAC TTC ATC CGT GCC CTG CCC AGC GCC		1737
Ala Asp Gln Asp Ile Asp Asn Tyr Phe Ile Arg Ala Leu Pro Ser Ala		
265 270 275		
GGT ACC ACC TCG TTC GAC GGC GGC ATC AAC TCG GCT ATC CTG CGC TAC		1785
Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr		
280 285 290		
TCT GGT GCC TCC GAG GTT GAC CCG ACG ACC ACG GAG ACC ACG AGC GTC		1833
Ser Gly Ala Ser Glu Val Asp Pro Thr Thr Glu Thr Thr Ser Val		
295 300 305 310		

CTC CCC CTC GAC GAG GCG AAC CTC GTG CCC CTT GAC AGC CCC GCT GCT Leu Pro Leu Asp Glu Ala Asn Leu Val Pro Leu Asp Ser Pro Ala Ala 315 320 325	1881
GTACGTCGTA TTCTGCGCTT GCAAGGATCG CACATACTAA CATGCTCTTG TAG CCC Pro	1937
GGT GAC CCC AAC ATT GGC GGT GTC GAC TAC GCG CTG AAC TTG GAC TTC Gly Asp Pro Asn Ile Gly Gly Val Asp Tyr Ala Leu Asn Leu Asp Phe 330 335 340	1985
AAC TTC GAT GGC ACC AAC TTC TTC ATC AAC GAC GTC TCC TTC GTG TCC Asn Phe Asp Gly Thr Asn Phe Phe Ile Asn Asp Val Ser Phe Val Ser 345 350 355	2033
CCC ACG GTC CCT GTC CTC CTC CAG ATT CTT AGC GGC ACC ACC TCC GCG Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Thr Thr Ser Ala 360 365 370 375	2081
GCC GAC CTT CTC CCC AGC GGT AGT CTC TTC GCG GTC CCG TCC AAC TCG Ala Asp Leu Leu Pro Ser Gly Ser Leu Phe Ala Val Pro Ser Asn Ser 380 385 390	2129
ACG ATC GAG ATC TCG TTC CCC ATC ACC GCG ACG AAC GCT CCC GCC GCG Thr Ile Glu Ile Ser Phe Pro Ile Thr Ala Thr Asn Ala Pro Gly Ala 395 400 405	2177
CCG CAT CCC TTC CAC TTG CAC GGT GTACGTGTCC CATCTCATAT GCTACGGAGC Pro His Pro Phe His Leu His Gly 410 415	2231
TCCACGCTGA CCGCCCTATA G CAC ACC TTC TCT ATC GTT CGT ACC GCC GGC His Thr Phe Ser Ile Val Arg Thr Ala Gly 420 425	2282
AGC ACG GAT ACG AAC TTC GTC AAC CCC GTC CGC CGC GAC GTC GTG AAC Ser Thr Asp Thr Asn Phe Val Asn Pro Val Arg Arg Asp Val Val Asn 430 435 440	2330
ACC GGT ACC GTC GGC GAC AAC GTC ACC ATC CGC TTC ACG GTACGCAGCA Thr Gly Thr Val Gly Asp Asn Val Thr Ile Arg Phe Thr 445 450	2379
CTCTCCTAAC ATTCCCCACTG CGCGATCACT GACTCCTCGC CCACAG ACT GAC AAC Thr Asp Asn 455	2434
CCC GGC CCC TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC TTG GAG GCC Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala 460 465 470	2482
GGT TTC GCC ATC GTC TTC AGC GAG GAC ACC GCC GAC GTC TCG AAC ACG Gly Phe Ala Ile Val Phe Ser Glu Asp Thr Ala Asp Val Ser Asn Thr 475 480 485	2530
ACC ACG CCC TCG GTACGTTGTG CTCCCGTGCC CATCTCCGCG CGCCTGACTA Thr Thr Pro Ser 490	2582
ACGAGCACCC CTTACAG ACT GCT TGG GAA GAT CTG TGC CCC ACG TAC AAC Thr Ala Trp Glu Asp Leu Cys Pro Thr Tyr Asn 495 500	2632
GCT CTT GAC TCA TCC GAC CTC TAATCGGTTA AAAGGGTCGC TCGCTACCTT Ala Leu Asp Ser Ser Asp Leu 505 510	2683

AGTAGGTAGA CTTATGCACC GGACATTATC TACAATGGAC TTTAATTGGA GTTAACGGCC	2743
GTTATACATA CGCGCACGTA GTATAAAGGT TCTCTGGATT GGTCGGACCT ACAGACTGCA	2803
ATTTTCGTGA CCTATCAACT GTATATTGAA GCACGACAGT GAATGGAAAT AGAGACA	2860

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 511 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile His Ser	
1 5 10 15	
Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile	
20 25 30	
Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu	
35 40 45	
Ala Asn Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys Gly Asp	
50 55 60	
Asn Phe Gln Ile Asn Val Ile Asp Asn Leu Ser Asn Glu Thr Met Leu	
65 70 75 80	
Lys Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn	
85 90 95	
Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr Gly	
100 105 110	
Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala Gly Thr Phe	
115 120 125	
Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly	
130 135 140	
Pro Met Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp Leu Tyr Asp	
145 150 155 160	
Val Asp Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp Tyr His Thr	
165 170 175	
Ala Ala Ser Leu Gly Ala Ala Phe Pro Ile Gly Ser Asp Ser Thr Leu	
180 185 190	
Ile Asn Gly Thr Asp Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg	
195 200 205	
Tyr Arg Met Arg Leu Leu Ser Leu Ser Cys Asp Pro Asn Tyr Val Phe	
210 215 220	
Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Ala Asp Ala Val Asn	
225 230 235 240	
His Glu Pro Leu Thr Val Asp Ser Ile Gln Ile Tyr Ala Gly Gln Arg	
245 250 255	
Tyr Ser Phe Val Leu Thr Ala Asp Gln Asp Ile Asp Asn Tyr Phe Ile	
260 265 270	

Arg Ala Leu Pro Ser Ala Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn
 275 280 285
 Ser Ala Ile Leu Arg Tyr Ser Gly Ala Ser Glu Val Asp Pro Thr Thr
 290 295 300
 Thr Glu Thr Thr Ser Val Leu Pro Leu Asp Glu Ala Asn Leu Val Pro
 305 310 315 320
 Leu Asp Ser Pro Ala Ala Pro Gly Asp Pro Asn Ile Gly Gly Val Asp
 325 330 335
 Tyr Ala Leu Asn Leu Asp Phe Asn Phe Asp Gly Thr Asn Phe Phe Ile
 340 345 350
 Asn Asp Val Ser Phe Val Ser Pro Thr Val Pro Val Leu Leu Gln Ile
 355 360 365
 Leu Ser Gly Thr Thr Ser Ala Ala Asp Leu Leu Pro Ser Gly Ser Leu
 370 375 380
 Phe Ala Val Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile Thr
 385 390 395 400
 Ala Thr Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His
 405 410 415
 Thr Phe Ser Ile Val Arg Thr Ala Gly Ser Thr Asp Thr Asn Phe Val
 420 425 430
 Asn Pro Val Arg Arg Asp Val Val Asn Thr Gly Thr Val Gly Asp Asn
 435 440 445
 Val Thr Ile Arg Phe Thr Thr Asp Asn Pro Gly Pro Trp Phe Leu His
 450 455 460
 Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Phe Ser
 465 470 475 480
 Glu Asp Thr Ala Asp Val Ser Asn Thr Thr Thr Pro Ser Thr Ala Trp
 485 490 495
 Glu Asp Leu Cys Pro Thr Tyr Asn Ala Leu Asp Ser Ser Asp Leu
 500 505 510

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2925 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: *Polyporus pinsitus*
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 734..808
- (ix) FEATURE:
 - (A) NAME/KEY: intron
 - (B) LOCATION: 878..932
- (ix) FEATURE:
 - (A) NAME/KEY: intron

(B) LOCATION: 1051..1104

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 1219..1270

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 1336..1397

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 1713..7744

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2030..2085

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2308..2375

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2492..2569

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: join (733..809, 877..933, 1050..1105, 1218..1271,
1335..1398, 1712..1775, 2029..2086, 2307..2376, 2492..2570).
2542..2600).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTCATAACTC TTGCTTCTA GCATGGGGC TGCGCACACC TGACAGACCC TTGGGAGGC	60
GAACCTGAAT GCAGCGTACT CTATCNCACC TCCAGGAAAG GTAGGGATGG ACNCCGTGCA	120
CCAACAACTG TCTCTCCACC AGCAACCATC CCTTGGATAT GTCTCCACAC ACCCGGTGTC	180
TACAAGCGGG GATCTGTGCT GGTGAAGTGC TGTCTCCGGA CGGGCGGCGG CGAGCGACCA	240
GAACCCGAAC CAGTGCTAGT GCCCGACACC CGCGAGACAA TTGTGCAGGG TGAGTTATAT	300
TCTTCGTGAG ACGGCGCTGC GCGTCGGCAC TGAAAGCGTC GCAGTTAGGT GATGCAGCGG	360
TCCCGCTAT TTTGACGTC TGGCAGCTAT CCTAAGCCGC GCCTCCATAC ACCCCAGGCG	420
CTCTCGTTTG CTATAGGTAT AAATCCCTCA GCTTCAGAGC GTCGATCCTC ATCCCACACG	480
ACACCCGTTT CAGTCTCTC GTAGCCGATT CCCTAGCCGC CCAGCCTCCG CTTTCGTTTT	540
CAAC ATG GGC AAG TAT CAC TCT TTT GTG AAC GTC GTC GCC CTT AGT CTT Met Gly Lys Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu	589
1 5 10 15	
TCT TTG AGC GGT CGT GTG TTC GGC GCC ATT GGG CCC GTC ACC GAC TTG Ser Leu Ser Gly Arg Val Phe Gly Ala Ile Gly Pro Val Thr Asp Leu	637
20 25 30	
ACT ATC TCT AAC GCC GAT GTT ACG CCT GAC GGC ATT ACT C3T GCT GCT Thr Ile Ser Asn Ala Asp Val Thr Pro Asp Gly Ile Thr Arg Ala Ala	685
35 40 45	
GTC CTC GCG GGC GTT TTC CCC GGG CCC CTC ATT ACC GGC AAC AAG Val Leu Ala Gly Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys	733
50 55 60	
GTGAGCCGCG AACCTTCTA CTAGCGCGT CGTACGGTGC ACCGTTACTG AAGCCACACT	793

TTGCGCTGTC AACAG GGG GAT GAA TTC CAG ATC AAT GTC ATC GAC AAC CTG Gly Asp Glu Phe Gln Ile Asn Val Ile Asp Asn Leu 65 70 75	844
ACC AAC GAG ACC ATG TTG AAG TCG ACC ACA ATC GTAAGGTGCT TGCTCCATA Thr Asn Glu Thr Met Leu Lys Ser Thr Thr Ile 80 85	897
ATTAAGCCCG TCGCTGACTC GAAGTTTATC TGTAG CAC TGG CAT GGT ATC TTC His Trp His Gly Ile Phe 90	950
CAG GCC GGC ACC AAC TGG GCA GAC GGC GCG GCC TTC GTG AAC CAG TGC Gln Ala Gly Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys 95 100 105	998
CCT ATC GCC ACG GGA AAC TCG TTC TTG TAC GAC TTC ACC GTT CCT GAT Pro Ile Ala Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp 110 115 120	1046
CAA GCC GTACGTTTAT ACACTTCCCT TTCTGCGGCA TACTCTGACG CGCCGCTGGA Gln Ala 125	1102
TCAG GGC ACC TTC TGG TAC CAC AGC CAC CTG TCC ACC CAG TAC TGT GAC Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp 130 135 140	1151
GGC CTG CGC GGT CCT CTT GTG GTC TAC GAC CCC GAC GAT CCC AAC GCG Gly Leu Arg Gly Pro Leu Val Val Tyr Asp Pro Asp Asp Pro Asn Ala 145 150 155	1199
TCT CTT TAC GAC GTC GAT GAC GTAAGCAGGC TACTTGTGGA CTTGTATGGA Ser Leu Tyr Asp Val Asp Asp 160	1250
TGTATCTCAC GCTCCCTAC AG GAT ACT ACG GTT ATT ACG CTT GCG GAC TGG Asp Thr Thr Val Ile Thr Leu Ala Asp Trp 165 170	1302
TAC CAC ACT GCG GCG AAG CTG GGC CCT GCC TTC CCC GTGAGTCTAC Tyr His Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro 175 180 185	1348
TCTTCCTCGT GTGTTAACAT AGGTGACGGC CGCTGATACG AGAGCTACCA G GCG GGT Ala Gly	1405
CCG GAT AGC GTC TTG ATC AAT GGT CTT GGT CGG TTC TCC GGC GAT GGT Pro Asp Ser Val Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly 190 195 200	1453
GGA GGA GCG ACA AAC CTC ACC GTG ATC ACC GTC ACG CAA GGC AAA CGG Gly Gly Ala Thr Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg 205 210 215 220	1501
G TGAGTCCGC CCTGAGCTGG CCTCAATAGC GATATTGACG AGTCCATGCC CTCCCAG	1558
TAC CGC TTC CGC CTT GTG TCG ATC TCG TGC GAC CCC AAC TTC ACG TTC Tyr Arg Phe Arg Leu Val Ser Ile Ser Cys Asp Pro Asn Phe Thr Phe 225 230 235	1606
TCG ATC GAC GGG CAC AAC ATG ACC ATC ATC GAG GTG GAC GGT GTC AAC Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Val Asp Gly Val Asn 240 245 250	1654
CAC GAG GCC TTG GAC GTC GAC TCC ATT CAG ATT TTT GCG GGG CAG CGG His Glu Ala Leu Asp Val Asp Ser Ile Gln Ile Phe Ala Gly Gln Arg 255 260 265	1702

TAC TCC TTC ATC GTACGTTCCC TTGCCCTCGT GCTATATCCG CCCGTCTGCT Tyr Ser Phe Ile 270	1754
CACAGAGGCT TCTATATCGC AG CTC AAC GCC AAC CAG TCC ATC GAC AAC Leu Asn Ala Asn Gln Ser Ile Asp Asn 275 280	1803
TAC TGG ATC CGC GCG ATC CCC AAC ACC GGT ACC ACC GAC ACC ACG GGC Tyr Trp Ile Arg Ala Ile Pro Asn Thr Gly Thr Thr Asp Thr Thr Gly 285 290 295	1851
GGC GTG AAC TCT GCT ATT CTT CGC TAC GAC ACC GCA GAA GAT ATC GAG Gly Val Asn Ser Ala Ile Leu Arg Tyr Asp Thr Ala Glu Asp Ile Glu 300 305 310	1899
CCT ACG ACC AAC GCG ACC ACC TCC GTC ATC CCT CTC ACC GAG ACG GAT Pro Thr Thr Asn Ala Thr Ser Val Ile Pro Leu Thr Glu Thr Asp 315 320 325	1947
CTG GTG CCG CTC GAC AAC CCT GCG GCT CCC GGT GAC CCC CAG GTC GGC Leu Val Pro Leu Asp Asn Pro Ala Ala Pro Gly Asp Pro Gln Val Gly 330 335 340 345	1995
GGT GTT GAC CTG GCT ATG AGT CTC GAC TTC TCC TTC GTGAGTCCCA Gly Val Asp Leu Ala Met Ser Leu Asp Phe Ser Phe 350 355	2041
CAGCACTCCG CGCCATTTCG CTTATTTACG CAGGAGTATT GTTCAG AAC GGT TCC Asn Gly Ser 360	2096
AAC TTC TTT ATC AAC AAC GAG ACC TTC GTC CCG CCC ACA GTT CCC GTG Asn Phe Phe Ile Asn Asn Glu Thr Phe Val Pro Pro Thr Val Pro Val 365 370 375	2144
CTC CTG CAG ATT TTG AGT GGT GCG CAG GAC GCG GCG AGC CTG CTC CCC Leu Leu Gln Ile Leu Ser Gly Ala Gln Asp Ala Ala Ser Leu Leu Pro 380 385 390	2192
AAC GGG AGT GTC TAC ACA CTC CCT TCG AAC TCG ACC ATT GAG ATC TCG Asn Gly Ser Val Tyr Thr Leu Pro Ser Asn Ser Thr Ile Glu Ile Ser 395 400 405	2240
TTC CCC ATC ATC ACC ACC GAC GGT GTT CTG AAC GCG CCC GGT GCT CCG Phe Pro Ile Ile Thr Thr Asp Gly Val Leu Asn Ala Pro Gly Ala Pro 410 415 420	2288
CAC CCG TTC CAT CTC CAC GGC GTCAGTCCCTT GCTTTCCCTCA GTGCCTCGCT His Pro Phe His Leu His Gly 425 430	2339
TCCACGACGT CCACTGATCC CACACATCCC ATGTGCAG CAC ACC TTC TCG GTG His Thr Phe Ser Val 435	2392
GTG CGC AGC GCC GGG AGC TCG ACC TTC AAC TAC GCC AAC CCA GTC CGC Val Arg Ser Ala Gly Ser Ser Thr Phe Asn Tyr Ala Asn Pro Val Arg 440 445 450	2440
CGG GAC ACC GTC AGT ACT GGT AAC TCT GGC GAC AAC GTC ACT ATC CGC Arg Asp Thr Val Ser Thr Gly Asn Ser Gly Asp Asn Val Thr Ile Arg 455 460 465	2488
TTC ACG GTACGTCCTTC TCCGGAGCCC TCCCACCCGT GTGTCCGCTG AGCGCTGAAC Phe Thr 470	2544
ACCGCCCACC GTGCTGCTGC TGCGCAG ACC GAC AAC CCA GGC CCG TGG TTC	2595

Thr Asp Asn Pro Gly Pro Trp Phe 475	
CTC CAC TGC CAC ATC GAC TTC CAC CTG GAG GCC GGC TTC GCC ATC GTC Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val 480 485 490	2643
TGG GGG GAG GAC ACT GCG GAC ACC GCG TCC GCG AAT CCC GTT CCT Trp Gly Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro 495 500 505	2688
GTACGTCGTG CCTGCTGAGC TCTTGTGCC CGAACAGGGT GCTGATCGTG CCTTCCTCCG	2748
TGCAG ACG GCG TGG AGC GAT TTG TGC CCC ACT TAC GAT GCT TTG GAC TCG Thr Ala Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser 510 515 520	2798
TCC GAC CTC TGATCGACAA GGCAATGAAGG CTGAAGCAGC TGCGGTCAAT Ser Asp Leu 525	2847
TCTCGAACAC ACTTIACTCG AACATTCAATT TTTCTTTGGC TCGGGATCGG AACAAATCAT GGGGGGGCCG GACCGTCT	2907 2925

(2) INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 527 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Polyporus pinsitus*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met	Gly	Lys	Tyr	His	Ser	Phe	Val	Asn	Val	Val	Ala	Leu	Ser	Leu	Ser
1					5				10				15		
Leu	Ser	Gly	Arg	Val	Phe	Gly	Ala	Ile	Gly	Pro	Val	Thr	Asp	Leu	Thr
			20					25				30			
Ile	Ser	Asn	Ala	Asp	Val	Thr	Pro	Asp	Gly	Ile	Thr	Arg	Ala	Ala	Val
			35				40			45					
Leu	Ala	Gly	Gly	Val	Phe	Pro	Gly	Pro	Leu	Ile	Thr	Gly	Asn	Lys	Gly
			50			55			60						
Asp	Glu	Phe	Gln	Ile	Asn	Val	Ile	Asp	Asn	Leu	Thr	Asn	Glu	Thr	Met
65					70			75			80				
Leu	Lys	Ser	Thr	Thr	Ile	His	Trp	His	Gly	Ile	Phe	Gln	Ala	Gly	Thr
			85				90				95				
Asn	Trp	Ala	Asp	Gly	Ala	Ala	Phe	Val	Asn	Gln	Cys	Pro	Ile	Ala	Thr
	100					105						110			
Gly	Asn	Ser	Phe	Leu	Tyr	Asp	Phe	Thr	Val	Pro	Asp	Gln	Ala	Gly	Thr
	115					120			125						
Phe	Trp	Tyr	His	Ser	His	Leu	Ser	Thr	Gln	Tyr	Cys	Asp	Gly	Leu	Arg
	130					135			140						
Gly	Pro	Leu	Val	Val	Tyr	Asp	Pro	Asp	Asp	Pro	Asn	Ala	Ser	Leu	Tyr
	145				150			155			160				

Asp Val Asp Asp Asp Thr Thr Val Ile Thr Leu Ala Asp Trp Tyr His
 165 170 175
 Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro Ala Gly Pro Asp Ser Val
 180 185 190
 Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly Gly Ala Thr
 195 200 205
 Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg Tyr Arg Phe Arg
 210 215 220
 Leu Val Ser Ile Ser Cys Asp Pro Asn Phe Thr Phe Ser Ile Asp Gly
 225 230 235 240
 His Asn Met Thr Ile Ile Glu Val Asp Gly Val Asn His Glu Ala Leu
 245 250 255
 Asp Val Asp Ser Ile Gln Ile Phe Ala Gly Gln Arg Tyr Ser Phe Ile
 260 265 270
 Leu Asn Ala Asn Gln Ser Ile Asp Asn Tyr Trp Ile Arg Ala Ile Pro
 275 280 285
 Asn Thr Gly Thr Thr Asp Thr Thr Gly Gly Val Asn Ser Ala Ile Leu
 290 295 300
 Arg Tyr Asp Thr Ala Glu Asp Ile Glu Pro Thr Thr Asn Ala Thr Thr
 305 310 315 320
 Ser Val Ile Pro Leu Thr Glu Thr Asp Leu Val Pro Leu Asp Asn Pro
 325 330 335
 Ala Ala Pro Gly Asp Pro Gln Val Gly Gly Val Asp Leu Ala Met Ser
 340 345 350
 Leu Asp Phe Ser Phe Asn Gly Ser Asn Phe Phe Ile Asn Asn Glu Thr
 355 360 365
 Phe Val Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala
 370 375 380
 Gln Asp Ala Ala Ser Leu Leu Pro Asn Gly Ser Val Tyr Thr Leu Pro
 385 390 395 400
 Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile Ile Thr Thr Asp Gly
 405 410 415
 Val Leu Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His
 420 425 430
 Thr Phe Ser Val Val Arg Ser Ala Gly Ser Ser Thr Phe Asn Tyr Ala
 435 440 445
 Asn Pro Val Arg Arg Asp Thr Val Ser Thr Gly Asn Ser Gly Asp Asn
 450 455 460
 Val Thr Ile Arg Phe Thr Thr Asp Asn Pro Gly Pro Trp Phe Leu His
 465 470 475 480
 Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Trp Gly
 485 490 495
 Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro Thr Ala Trp
 500 505 510
 Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser Ser Asp Leu
 515 520 525

Applicant's or agent's file
reference number

4185.204-WO

International application
to be assigned

PCT/US 95/07536

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description
on page 55, line 4

B. IDENTIFICATION OF

Further deposits are identified on an additional sheet

Name of depository institution

Agricultural Research Service Patent Culture Collection (NRRL)

Address of depository institution (*including postal code and country*)

Northern Regional Research Center
1815 University Street
Peoria, IL 61604, US

Date of deposit
May 25, 1995

Accession Number
NRRL B-21263

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) This information is continued on an additional sheet

In respect of those designations in which a European and/or Australia Patent is sought, during the pendency of the patent application, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rule 1991 No. 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indication listed below will be submitted to the International Bureau Later (*specify the general nature of the indications e.g. "Accession Number of Deposit"*)

For receiving Office use only

This sheet was received with the international application

Authorized officer Doris L. Brock *DLB*
PCT International Division

For International Bureau use only

This sheet was received with the International Bureau on:

Authorized officer

Applicant's or agent's file reference number	4185.204-WO	International application N to be assigned PCT/US 95/07536
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> , line <u>6</u>	
B. IDENTIFICATION OF <input checked="" type="checkbox"/> Further deposits are identified on an additional sheet	
Name of depository institution Agricultural Research Service Patent Culture Collection (NRRL)	
Address of depository institution (including postal code and country) Northern Regional Research Center 1815 University Street Peoria, IL 61604, US	
Date of deposit May 25, 1995	Accession Number NRRL B-21268
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <input type="checkbox"/> This information is continued on an additional sheet	
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4185.204-WO

International application N
to be assigned

PCT/US 95/07536

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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A. The indications made below relate to the microorganism referred to in the description
on page 55, line 11

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NRRL B-21264

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A. The indications made below relate to the microorganism referred to in the description
on page 55, line 14

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Date of deposit
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Address of depository institution (<i>including postal code and country</i>) Northern Regional Research Center 1815 University Street Peoria, IL 61604, US		
Date of deposit <u>May 25, 1995</u>	Accession Number <u>NRRL B-21266</u>	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (<i>if the indications are not for all designated States</i>)		
E. SEPARATE FURNISHING OF INDICATIONS (<i>leave blank if not applicable</i>)		
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A. The indications made below relate to the microorganism referred to in the description on page <u>55</u> , line <u>18</u>		
B. IDENTIFICATION OF		Further deposits are identified on an additional sheet <input type="checkbox"/>
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Address of depository institution (<i>including postal code and country</i>) Northern Regional Research Center 1815 University Street Peoria, IL 61604, US		
Date of deposit May 25, 1995	Accession Number	NRRL B-21267
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (<i>if the indications are not for all designated States</i>)		
E. SEPARATE FURNISHING OF INDICATIONS (<i>leave blank if not applicable</i>)		
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What we claim is:

1. A DNA construct containing a sequence encoding a *Polyporus* laccase.

5

2. The construct of Claim 1 which comprises a sequence encoding a *Polyporus pinsitus* laccase.

3. The construct of Claim 1 which comprises a nucleic acid 10 sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.

15

5. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 4.

20 6. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 3.

7. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID 25 NO. 6.

8. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 5.

30 9. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 8.

10. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 7.

11. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 10.

12. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 9.

10

13. The construct of Claim 1, which comprises the nucleic acid sequence selected from those contained in NRRL B-21263, 21264, 21265, 21266, 21267, and 21268.

15

14. A substantially pure *Polyporus* laccase enzyme.

15. The enzyme of Claim 14 which is a *Polyporus pinsitus* laccase.

20

16. The enzyme of Claim 14 which comprises the amino acid sequence selected from the group consisting of the sequences depicted in SEQ ID NOS. 4, 6, 8, and 10 or a sequence with at least about 80% homology thereto.

25

17. A recombinant vector comprising an DNA construct containing a sequence encoding a *Polyporus* laccase.

18. The vector of Claim 17 in which the construct is operably linked to a promoter sequence.

30

19. The vector of Claim 18 in which the promoter is a fungal or yeast promoter.

20. The vector of Claim 19 in which the promoter is the TAKA amylase promoter of *Aspergillus oryzae*.

21. The vector of Claim 18 in which the promoter is the 5 glucoamylase (*glaA*) promoter of *Aspergillus niger* or *Aspergillus awamori*.

22. The vector of Claim 17 which also comprises a selectable marker.

10

23. The vector of Claim 22 in which the selectable marker is selected from the group consisting of *amdS*, *pyrG*, *argB*, *niaD*, *sc*, *trpC* and *hygB*.

15

24. The vector of Claim 22 in which the selectable marker is the *amdS* marker of *Aspergillus nidulans* or *Aspergillus oryzae*, or the *pyrG* marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.

20

25. The vector of Claim 18 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the *amdS* or *pyrG* marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

25

26. A recombinant host cell comprising a heterologous DNA construct containing a sequence encoding a *Polyporus laccase*.

27. The cell of Claim 26 which is a fungal cell.

30

28. The cell of Claim 27 which is an *Aspergillus* cell.

29. The cell of Claim 26 in which the construct is integrated into the host cell genome.

30. The cell of Claim 26 in which the construct is contained on a vector.

5 31. The cell of Claim 26 which comprises a construct containing a sequence encoding an amino acid sequence selected from the group consisting of those depicted in SEQ ID NOS. 2, 4, 6, 8, and 10.

10 32. A method for obtaining a laccase enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Polyporus* laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.

15 33. A method for obtaining a laccase enzyme which comprises culturing a recombinant *Aspergillus* host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Polyporus*-like laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.

34. A *Polyporus* enzyme obtained by the method of Claim 33.

20 25 35. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Polyporus* laccase.

30 36. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Polyporus* laccase.

37. A method for oxidizing dyes or dye precursors which comprises contacting the dye or dye precursor with a *Polyporus* laccase.

5 38. A method for dyeing hair which comprises contacting a *Polyporus* laccase, in the presence or absence of at least one modifier, with at least one dye precursor, for a time and under conditions sufficient to permit oxidation of the dye precursor to a dye.

10

39. The method of claim 38 in which the dye precursor is selected from the group consisting of a diamine, aminophenol, and a phenol.

15 40. The method of claim 38, wherein the modifier, when used, is a meta-diamine, a meta-aminophenol or a polyphenol.

41. The method of claim 38 in which the dye precursor is a primary intermediate selected from the group consisting of 20 an ortho- or para-diamine or aminophenol.

42. The method of claim 38 in which more than one dye precursor is used.

25 43. The method of claim 38 in which more than one modifier is used.

44. The method of claim 38 in which both a primary intermediate and a modifier are used.

30

45. A dye composition comprising a *Polyporus* laccase combined with at least one dye precursor.

46. A dye composition comprising a *Polyporus* laccase combined with at least one primary intermediate and at least one modifier.

5 47. A container containing a dye composition comprising a *Polyporus* laccase and at least one dye precursor in an oxygen-free atmosphere.

10 48. The container of claim 47 which contains at least one primary intermediate dye precursor combined with at least one modifier.

15 49. A method of polymerizing or oxidizing a phenolic or aniline compound which comprises contacting the phenolic or aniline compound with a *Polyporus* laccase.

10	20	30	40	50	60	70
AGATTTCTGA CACCGGTGCA <u>ATCTTGACAC</u> TGTACCAACC GGGCAAGTCT CGTCCTTGGT TCTCGGGGAC						
80	90	100	110	120	130	140
TGGCGCCCGT CGCTACCCCT TGGTCATTCA CTCTACCAGA GCGCTGGCTT CGCCGAGGTA <u>TAAAGGATGT</u>						
150	160	170	180	190	200	210
TGGGGGACAC CCTCAACACC CCAACTCAAG CCCCACCTGA GCTTTGCGA GATCCTCCAC ATACCACTCA						
220	230	239	248	257	266	
> CTACTTTCAA GTTCTTCAAC ATG TCG AGG TTT CAC TCT CTT CTC GCT TTC GTC GTT Met Ser Arg Phe His Ser Leu Leu Ala Phe Val Val						
275	284	293	302	311	320	
GCT TCC CTT ACG GCT GTG GCC CAC GCT GGT ATC GGT CCC GTC GCC GAC CTA ACC Ala Ser Leu Thr Ala Val Ala His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr						
329	338	347	356	365	374	
ATC ACC AAC GCA GCG GTC AGC CCC GAC GGG TTT TCT CGC CAG GCC GTC GTC GTC Ile Thr Asn Ala Ala Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val						
383	392	401	410	423	433	
AAC GGC GGC ACC CCT GGC CCT CTC ATC ACC GGT AAC ATG GTTCGTCTCG GCTGGCACTA Asn Gly Gly Thr Pro Gly Pro Leu Ile Thr Gly Asn MET						
443	453	463	473	482	491	
<u>GGGGGTTGTA</u> TCGTTCTGA CCTTGTGGA G <u>GGG</u> GAT CGC TTC CAG CTC AAT GTC ATC Gly Asp Arg Phe Gln Leu Asn Val Ile						
500	509	518	527	543	553	
GAC AAC CTT ACC AAC CAC ACG ATG GTG AAG ACC ACG AGT ATT GTGAGCTGCT ATTCTCCGG Asp Asn Leu Thr Asn His Thr MET Val Lys Ser Thr Ser Ile						

563 573 583 592 601 610

ACGGGGCTTC ATTGTGCTAA TAATCGTCGT GTGCAG CAC TGG CAC GGT TTC TTC CAG AAG
His Trp His Gly Phe Phe Gln Lys

619 628 637 646 655 664

GTG ACC AAC TGG GCC GAC GGT CCC GCC TTC ATC AAC CAG TGC CCG ATC TCA TCT
Gly Thr Asn Trp Ala Asp Gly Pro Ala Phe Ile Asn Gln Cys Pro Ile Ser Ser

673 682 691 700 709 720

GTG CAC TCG TTC CTG TAC GAC TTC CAG GTT CCT GAC CAG GCT G GTAAGTACGG
Gly His Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly

730 740 750 760 770 779

TCGTTATGGA GTATACTGCG CATTGCTAAA CCACATGGTC AACAG GT ACC TTC TGG TAT
Thr Phe Trp Tyr

788 797 806 815 824 833

CAC AGT CAC TTG TCT ACG CAG TAC TGT GAT GGT TTG AGG GGT CCG TTC GTT GTT
His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val

842 851 860 869 878 889

TAC GAC CCC AAT GAC CCG GCC GAC CTG TAC GAC GTC GAC AAC G GTAAGGACGA
Tyr Asp Pro Asn Asp Pro Ala Ala Asp Leu Tyr Asp Val Asp Asn Asp

899 909 919 929 940 949

ATTCGAACCG TAAATACTTG CTTACTGATA CTTCTCGATG AATTAG AC GAC ACT GTC ATT
Asp Thr Val Ile

958 967 976 985 994 1009

ACC CTT GTG GAT TGG TAC CAC GTC GCC GCG AAG CTG GGC GGG GCA TTC CC GTAAGTCCAT
Thr Leu Val Asp Trp Tyr His Val Ala Ala Lys Leu Gly Pro Ala Phe Pro

FIG.1B

1019	1029	1039	1049	1060	1069
GAGTATTCTG CTGTTGAATC <u>TGTCTTA</u> ACT GTGCATATCA G T CTC GGC GCC GAC GCC ACC Leu Gly Ala Asp Ala Thr					
1078	1087	1096	1105	1114	1123
<u>CTC</u> ATC AAC GGT AAG GGA CGC TCC CCC AGC ACG ACC ACC GCG GAC CTC TCA GTT Leu Ile Asn Gly Lys Arg Ser Pro Ser Thr Thr Thr Ala Asp Leu Ser Val					
1132	1141		1156	1166	1176
<u>ATC</u> AGC GTC ACC CCG GGT AAA CG GTATGCTATA TCTTATCTTA TCTGATGGCA TTTCTCTGAG Ile Ser Val Thr Pro Gly Lys Arg					
1196	1207	1216	1225	1234	
ACATTCTCCA G C TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC GAC CCC AAC TAC Tyr Arg Phe Arg Leu Val Ser Leu Ser Cys Asp Pro Asn Tyr					
1243	1252	1261	1270	1279	1288
<u>ACG</u> TTC AGC ATC GAT GGT CAC AAC ATG ACG ATC ATC GAG ACC GAC TCA ATC AAC Thr Phe Ser Ile Asp Gly His Asn MET Thr Ile Ile Glu Thr Asp Ser Ile Asn					
1297	1306	1315	1324	1333	1342
<u>ACC</u> GCG CCC CTC GTC GTC GAC TCC ATT CAG ATC TTC GCC CCC CAG CGT TAC TGC Thr Ala Pro Leu Val Val Asp Ser Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser					
1351	1364	1374	1384	1394	1404
<u>TTC</u> GTG GTAAGTTCGA TTCATCCTCT AACGTTGGTC GCTGTTAGTC ATCGTATGGT CATGTAG Phe Val					
1414	1423	1432	1441	1450	1459
<u>CTC</u> GAG GCC AAC CAG GCC GTC GAC AAC TAC TGG ATT CGC GCC AAC CCG AAC TTC Leu Glu Ala Asn Gln Ala Val Asp Asn Tyr Trp Ile Arg Ala Asn Pro Asn Phe					

FIG.1C

1468	1477	1486	1495	1504	1513
GGT AAC GTC GGG	TTC ACC GGC GGC	ATT AAC TCG GCT	ATC CTC CGC TAC GAT GGT		
Gly Asn Val Gly Phe Thr Gly Gly	Ile Asn Ser Ala Ile Leu Arg Tyr Asp Gly				
1522	1531	1540	1549	1558	1567
GCC GCT GCC GTG GAG CCC ACC	ACA ACG CAA ACC ACG TCG ACT	GCG CCG CTC AAC			
Ala Ala Ala Val Glu Pro Thr Thr Gln Thr Thr Ser Thr Ala Pro Leu Asn					
1576	1585	1594	1603	1619	1629
GAG GTC AAC CTG CAC CCG CTG GTT ACC ACC GCT GTG	GTATGTAATA TTGTCGGTAA				
Glu Val Asn Leu His Pro Leu Val Thr Thr Ala Val					
1639	1649	1659	1669	1678	1687
TGTAATACAT TGTGCTGAC	CTCGACCCCC ACAG CCT GGC TCG CCC GTC GCT GGT				
	Pro Gly Ser Pro Val Ala Gly Gly				
1696	1705	1714	1723	1732	1741
GTC GAC CTG GCC ATC AAC ATG GCG	TTC AAC TTC AAC GGC ACC AAC TTC TTC ATC				
Val Asp Leu Ala Ile Asn MET Ala Phe Asn Phe Asn Gly Thr Asn Phe Phe Ile					
1750	1759	1768	1777	1786	1795
AAC GGC ACG TCT TTC ACG CCC CCG ACC GTG CCT GTC CTG CTC CAG ATC ATC AGC					
Asn Gly Thr Ser Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile Ile Ser					
1804	1813	1822	1831	1840	1849
GGC GCG CAG AAC GCG CAG GAC CTC CTG CCC TCC GGT AGC GTC TAC TCG CTT CCC					
Gly Ala Gln Asn Ala Gln Asp Leu Leu Pro Ser Gly Ser Val Tyr Ser Leu Pro					
1858	1867	1876	1885	1894	1903
TCG AAC GCC GAC ATC GAG ATC TCC TTC CCC GCC ACC GCC GCC CCC GGT GCG					
Ser Asn Ala Asp Ile Glu Ile Ser Phe Pro Ala Thr Ala Ala Pro Gly Ala					

FIG.1D

1912	1921	1930	1939	1948	1957
CCC CAC CCC TTC CAC TTG CAC GGG CAC GCG TTC GCG GTC GTC CGC AGC GCC GGC					
Pro His Pro Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly					
1966	1975	1984	1993	2002	2011
AGC ACG GT TAC AAC TAC GAC AAC CCC ATC TTC CGC GAC TC GTC AGC ACG GGC					
Ser Thr Val Tyr Asn Tyr Asp Asn Pro Ile Phe Arg Asp Val Val Ser Thr Gly					
2020	2029	2038	2047	2056	2065
ACG CCT GCG GCC GGT GAC AAC GTC ACC ATC CGC TTC CGC ACC GAC AAC CCC GGC					
Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Arg Thr Asp Asn Pro Gly					
2074	2083	2092	2101	2110	2119
CCG TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC CTC GAG GCC GGC TTC GCC GTC					
Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Val					
2128	2137	2146	2155	2164	2173
TG TG GCG GAG GAC ATC CCC GAC GTC GCG TCG GCG AAC CCC GTC CCC CAG GGC					
Val Phe Ala Glu Asp Ile Pro Asp Val Ala Ser Ala Asn Pro Val Pro Gln Ala					
2182	2191	2200	2209	2218	2231
TGG TCC GAC CTC TGT CCG ACC TAC GAC GCG CTC GAC CCG AGC GAC CAG TAAATGGCTT					
Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Pro Ser Asp Gln					
2241	2251	2261	2271	2281	2291
GCGCCGGTCC ATGATAGGAT ATGGACGGTG AGTTCCCACT TGCAATACGG ACTCTCGCCT CATTATGGTT					
2311	2321	2331	2341	2351	2361
ACACACTCGC TCTGGATCTC TCGCCTGTCC ACAGAACAAA CTTGTATAAT TCGCTTAATC GTTGAACAA					
2381	2391	2401	2411		
ATGGAATATT GGGGTACTAT GCACGCATCT CGCTGGGTCA GCTTTCGT					

10	20	30	40	50	60	70
GCGGGCGACA AACCGTGGGA GCCAACACAC TCCCCTCCAC TCTCACACTG CCCAGATTCC CGCGACCGCC						
80	90	100	110	120	130	140
GCCTTTCAAGG CCCAAACAGA TCTGGCAGGT TTGGATGGGG CACGGGGGGG TCCCTGGGG ATTCAATTGT						
150	160	170	180	190	200	210
GCGCCAGTCG GGCATCCGGA TGGCTCTACC AGCGCCGTTG ACTGGAAGAG AACACCGAGG TCATGCATTG						
220	230	240	250	260	270	280
TGGCCAAGTG CGGCCAAAGG ACCGCTCGCT GGTGCGGATA CTTAAAGGGC GGCGGGGA GGCGCTGTCTA						
290	300	310	320	330	340	350
CCAAGCTCAA GCTCGCCCTTG GGTCCCAGT CTCCGCCACC CTCCCTTTCC CCCACACAGT CGCTCCATAG						
360	369	378	387	396	405	
> CACCGTCGGC GCC ATG GGT CTG CAG CGA TTC AGC TTC TTC GTC ACC CTC GCG CTC MET Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu						
414	423	432	441	450	459	
GTC CCT CGC TCT CTT GCA GCC ATC GGG CCG GTG GCG AGC CTC GTC GTC GCG AAC Val Ala Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala Asn						
468	477	486	495	504	513	
GCC CCC GTC TCG CCC GAC GGC TTC CTT CGG GAT GCC ATC GTG GTC AAC GGC GTG Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val Asn Gly Val						
522	531	540	553	563	573	
GTC CCT TCC CCC CTC ATC ACC GGG AAG AAG GTCGGGGTGT TCGTCGTGCT CCTACTCCT Val Pro Ser Pro Leu Ile Thr Gly Lys Lys						

FIG.2A

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583	592	601	610	619	628
TGCTGACAGC GATCTACAG <u>GGA</u> <u>GAC</u> <u>CGC</u> <u>GTC</u> <u>CAG</u> <u>CTC</u> <u>AAC</u> <u>GTC</u> <u>GTC</u> <u>GAC</u> <u>ACC</u> <u>TTG</u> Gly Asp Arg Phe Gln Leu Asn Val Val Asp Thr Leu					
637	646	655	671	681	691
ACC AAC CAC AGC ATG CTC AAG TCC ACT ACT ATC <u>GTAAGTGTGA</u> CGATCCGAAT GTGACATCAA Thr Asn His Ser MET Leu Lys Ser Thr Ser Ile					
701	711	721	730	739	748
TCGGGGCTAA TTAACCGCGC ACAG <u>CAC</u> <u>TGG</u> <u>CAC</u> <u>GGC</u> <u>TTC</u> <u>TTC</u> <u>CAG</u> <u>GCA</u> <u>GCC</u> <u>ACC</u> <u>AAC</u> His Trp His Gly Phe Phe Gln Ala Gly Thr Asn					
757	766	775	784	793	802
TGG GCA GAA GGA CCC GCG TTC GTC AAC CAG TGC CCT ATT GCT TCC GGG CAT TCA Trp Ala Glu Gly Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser					
811	820	829	846	856	
TTC CTG TAC GAC TTC CAT GTG CCC GAC CAG GCA G <u>GTAAGCAGGA</u> TTTTCTGGGG Phe Leu Tyr Asp Phe His Val Pro Asp Gln Ala Gly					
866	876	886	896	905	914
TCCCCGTGTC ATGCAATGTT CTCATGCTCC GACGTGATCG ACAG <u>GG</u> <u>ACG</u> <u>TTC</u> <u>TGG</u> <u>TAC</u> <u>CAC</u> Thr Phe Trp Tyr His					
923	932	941	950	959	968
AGT CAT CTG TCT ACG CAG TAC TGT GAC GGG CTG CGG GGG CCG TTC GTC GTG TAC Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val Tyr					
977	986	995	1004	1013	1024
GAC CCC AAG GAC CCG CAC GCC AGC CGT TAC GAT GTT GAC AAT G <u>GTACGTGCCG</u> Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val Asp Asn Glu					

FIG.2B

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1034	1044	1054	1064	1075	1084
CACGGAGTAT ATCACACAGC ATGCGTTGAC GTCGGGCCAA CAGAG AGC ACG GTC ATC ACG Ser Thr Val Ile Thr					
1093	1102	1111	1120	1129	1141
TTG ACC GAC TGG TAC CAC ACC GCT GCC CGG CTC GGT CCC AAG TTC CC GTAAGCTCGC Leu Thr Asp Trp Tyr His Thr Ala Ala Arg Leu Gly Pro Lys Phe Pro					
1151	1161	1171	1181	1190	1199
AATGGCTTAG TGTCACAGG TTCTTGCTT ATGTTGCTTC GATAG A CTC GGC GCG GAC GCC Leu Gly Ala Asp Ala					
1208	1217	1226	1235	1244	1253
ACG CTC ATC AAC GGT CTG GGG CGG TCT GCC TCC ACT CCC ACC GCT GCG CTT GCC Thr Leu Ile Asp Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala					
1262	1271	1280	1292	1302	1312
GTC ATC AAC GTC CAG CAC GGA AAG CG GTGAGCATTG TCTTGTATCC CATTCAATG Val Ile Asn Val Gln His Gly Lys Arg					
1322	1332	1341	1351	1360	1369
CTTTGTGCTG ACCTATCGGA ACCGGCGAG C TAC CGC TTC CGT CTC GTT TCG ATC TCG Tyr Arg Phe Arg Leu Val Ser Ile Ser					
1378	1387	1396	1405	1414	1423
TGT GAC CCG AAC TAC ACG TTC AGC ATC GAC GGG CAC AAC CTG ACC GTC ATC GAG Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr Val Ile Glu					
1432	1441	1450	1459	1468	1477
GTC GAC GGC ATC AAT AGC CAG CCT CTC CTT GTC GAC TCT ATC CAG ATC TTC GCC Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser Ile Gln Ile Phe Ala					
1486	1495	1508	1518	1528	1538
GCA CAG CGC TAC TCC TTC GTG GTAAAGTCCTG GCTTGTGAT GCTCCAAAGT GGCCTCACTC Ala Gln Arg Tyr Ser Phe Val					

FIG.2C
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1548	1559	1568	1577	1586		
ATATACTTTC GTTAG <u>TTG</u> AAT <u>GCG</u> AAT <u>CAA</u> <u>ACG</u> <u>GTG</u> <u>GGC</u> AAC <u>TAC</u> <u>TGG</u> <u>GTG</u> <u>CGT</u> Leu Asn Ala Asn Gln Thr Val Gly Asn Tyr Trp Val Arg						
1595	1604	1613	1622	1631	1640	
<u>GCG</u> AAC <u>CCG</u> AAC <u>TTC</u> <u>GGA</u> <u>ACG</u> <u>GTT</u> <u>GGG</u> <u>TTC</u> <u>GCC</u> <u>GGG</u> <u>GGG</u> <u>ATC</u> AAC <u>TCC</u> <u>GCC</u> <u>ATC</u> Ala Asn Pro Asn Phe Gly Thr Val Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile						
1649	1658	1667	1676	1685	1694	
<u>TTG</u> <u>CGC</u> <u>TAC</u> <u>CAG</u> <u>GGC</u> <u>GCA</u> <u>CCG</u> <u>GTC</u> <u>GCC</u> <u>GAG</u> <u>CCT</u> <u>ACC</u> <u>ACG</u> <u>ACC</u> <u>CAG</u> <u>ACG</u> <u>CCG</u> <u>TCG</u> Leu Arg Tyr Gln Gly Ala Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser						
1703	1712	1721	1730	1739	1748	1761
<u>GTG</u> <u>ATC</u> <u>CCG</u> <u>CTC</u> <u>ATC</u> <u>GAG</u> <u>ACG</u> AAC <u>TTG</u> <u>CAC</u> <u>CCG</u> <u>CTC</u> <u>GCG</u> <u>CGC</u> <u>ATG</u> <u>CCA</u> <u>GTG</u> <u>GTATGTCTCT</u> Val Ile Pro Leu Ile Glu Thr Asn Leu His Pro Leu Ala Arg MET Pro Val						
1771	1781	1791	1801	1811	1821	
TTTCTGATC ATCTGAGTTG CCCGTTCTTG ACCCCATTAT GTGTTACTAT CTAG <u>CCT</u> <u>GGC</u> <u>AGC</u> Pro Gly Ser						
1830	1839	1848	1857	1866	.	1882
<u>CCG</u> <u>ACA</u> <u>CCC</u> <u>GGG</u> <u>GCG</u> <u>GTC</u> <u>GAC</u> <u>AAG</u> <u>GCG</u> <u>CTC</u> <u>AAC</u> <u>CTC</u> <u>GCG</u> <u>TTT</u> <u>AAC</u> <u>TTC</u> <u>GTAACTATCT</u> Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu Ala Phe Asn Phe						
1892	1902	1912	1922	1931	1940	
CTACTACTT GGCTGGAGGC TGGTCGCTGA TCATACGGTG CTTCA <u>G</u> AAC <u>GGC</u> <u>ACC</u> AAC <u>TTC</u> Asn Gly Thr Asn Phe						
1949	1958	1967	1976	1985	1994	
<u>TTC</u> <u>ATC</u> <u>AAC</u> <u>AAC</u> <u>GCG</u> <u>ACT</u> <u>TTC</u> <u>ACG</u> <u>CCG</u> <u>CCG</u> <u>ACC</u> <u>GTC</u> <u>CCG</u> <u>GTA</u> <u>CTC</u> <u>CTC</u> <u>CAG</u> <u>ATT</u> Phe Ile Asn Asn Ala Thr Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile						

FIG.2D

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2003	2012	2021	2030	2039	2048
CTG AGC GGT GCG CAG ACC GCA CAA GAC	CTG CTC CCC GCA GGC TCT GTC TAC CCG				
Leu Ser Gly Ala Gln Thr Ala Gln Asp	Leu Leu Pro Ala Gly Ser Val Tyr Pro				
2057	2066	2075	2084	2093	2102
CTC CCG GCC CAC TCC ACC ATC GAG ATC ACG	CTG CCC GCG ACC GCC TTG GCC CCC				
Leu Pro Ala His Ser Thr Ile Glu Ile Thr	Leu Pro Ala Thr Ala Leu Ala Pro				
2111	2120	2129		2145	2155
GGT GCA CCG CAC CCC TTC CAC CTG CAC GGT	GTATGTTCCC CTGCCTTCCC TTCTTATCCC				
Gly Ala Pro His Pro Phe His Leu His Gly					
2175	2185	2195	2204	2213	2222
CGAACCACTG CTCACGTCCG TCCCCATCTAG CAC	GCC TTC GCG GTC GTT CGC AGC GCG				
His Ala Phe Ala Val Val Arg Ser Ala					
2231	2240	2249	2258	2267	2276
GGG AGC ACC ACG TAT AAC TAC AAC GAC CCC	ATC TTC CGC GAC GTC GTG AGC ACG				
Gly Ser Thr Thr Tyr Asn Tyr Asn Asp Pro	Ile Phe Arg Asp Val Val Ser Thr				
2285	2294	2303	2312	2321	2330
GGC ACG CCC GCC GCG GGC GAC AAC GTC ACG	ATC CGC TTC CAG ACC GAC AAC CCC				
Gly Thr Pro Ala Ala Gly Asp Asn Val Thr	Ile Arg Phe Gln Thr Asp Asn Pro				
2339	2348	2357	2366	2375	2384
GGC CCC TGG TTC CTC CAC TGG CAC ATC GAC	TTC CAC CTC GAC GCA GGC TTC GCG				
Gly Pro Trp Phe Leu His Cys His Ile Asp Phe	His Leu Asp Ala Gly Phe Ala				
2393	2402	2411	2420	2429	2438
ATC GTG TTC GCA GAG GAC GTT GCG GAC GTG	AAG GCG GCG AAC CCG GTT CCG AAC				
Ile Val Phe Ala Glu Asp Val Ala Asp Val Lys	Ala Ala Asn Pro Val Pro Lys				

FIG.2E

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2447	2456	2465	2474	2483	2499												
—	—	—	—	—	—>												
GCG	TGG	TCG	GAC	CTG	TGC	CCG	ATC	TAC	GAG	GGG	CTG	AGC	GAG	GCT	AAC	CAG	TGAGCGGAGG
Ala	Trp	Ser	Asp	Leu	Cys	Pro	Ile	Tyr	Asp	Gly	Leu	Ser	Glu	Ala	Asn	Gln	
2509	2519	2529	2539	2549	2559	2569											
CCGTGGTGTG	GAGCGTAAAG	CTCGCGCGTC	CACCTGGGGG	GTTGAAGGTG	TTCTGATTGA	AATGGTCTTT											
2579	2589	2599	2609	2619	2629	2639											
GGGTTTATTT	CTTGTATTTC	TAACCTGGTT	CTCTACGCAA	GGACCGAGGA	TTGTATAGGA	TGAAGTAACT											
2649	2659	2669	2679	2689													
TTCCTAATGT	ATTATGATAT	CAATTGACGG	AGGCATGGAC	TCCGAAGTGT													

FIG.2F

10 20 30 40 50 60 70
 TTTCCCGACT AAACCAATCT CAGNCCGCTT CCTCCTAGGG AACCGAGCGA TGTGGCGGCC CTCTCTATCC
 80 90 100 110 120 130 140
 AAGCTGTCCA TAAGAAGACG TTCAAATGCC GCAGCAAGCG AGGAAATAAG CATCTAACAG TGTTTTCC
 150 160 170 180 190 200 210
 ATAGTCGCAT TTGCCCCGCC TGTCGGACCG ACGCCCCTAG AGCGCTTGG GAAACGTCGC AAGTGGCGGG
 220 230 240 250 260 270 280
 TGTTATTCTGT GTAGACCGAGA CGGTATTTGT CTCATCATTC CCGTGCTTCA CGTTGACACA GCCCAAAGGT
 290 300 310 320 330 340 350
 CTATGTACGG CCCTTCACAT TCCCTGACAC ATTGACCGAA CCCTCGGTGC CCCTCCGACA GTGCCCTCGGT
 360 370 380 390 400 410 420
 TGTAGTATCG GGACGCCCTA GGATGCAAGA TTGGAAGTCA CCAAGGCCCG AAGGCTATAA AATACCGAGA
 430 440 450 460 470 480
 GGTCCCTACCA CTTCTGCATC TCCAGTCGCA GAGTTCCCTCT CCCTTGCCAG CCACAGCTCG AG
 491 500 509 518 527 536
 > ATG TCC TTC TCT AGC CTT CGC CGT GCC TTG GTC TTC CTG GGT GCT TCC AGC AGT
 MET Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala Cys Ser Ser
 545 554 563 572 581 590
 GCG CTG GCC TCC ATC GGC CCA GTC ACT GAG CTC GAC ATC GTT AAC AAG GTC ATC
 Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile Val Asn Lys Val Ile
 599 608 617 626 635 644
 GCC CCG GAT GGC GTC GCT CGT GAT ACA GTC CTC GCC GGG GGC ACC TTC CCG GGC
 Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu Ala Gly Gly Thr Phe Pro Gly
 653 662 675 685 695 705
 CCA CTC ATC ACA GGA AAG AAG GTATGCTAAG TAGTCCCCGCC CCCATCATCC TGTGGCTGAC
 Pro Leu Ile Thr Gly Lys Lys

FIG.3A

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715	726	735	744	753	
GTTCGACGCC GCCAG GGT GAC AAC TTC CGC ATC AAC GTC GTC GAC AAG TTG GTT Gly Asp Asn Phe Arg Ile Asn Val Val Asp Lys Leu Val					
762	771	780	789	799	819
AAC CAG ACT ATG CTG ACA TCC ACC ACC ATT GTATGTCACT AGCTCTCGCT ATCTCGAGAC Asn Gln Thr MET Leu Thr Ser Thr Thr Ile					
829	839	848	857	866	875
CCGCTGACCG ACAACATTC CCGTAG CAC TGG CAC GGG ATG TTC CAG CAT ACG ACG His Trp His Gly MET Phe Gln His Thr Thr					
884	893	902	911	920	929
AAC TGG GCG GAT GGT CCC GCC TTT GTG ACT CAA TGC CCT ATC ACC ACC GGT GAT Asn Trp Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Thr Thr Gly Asp					
938	947	956	965	976	986
GAT TTC CTG TAC AAC TTC CGC GTG CCC GAC CAG ACA G GTACGCCAAAG CCCAGCATGC Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr Gly					
996	1006	1016	1026	1035	1044
GTACTCAAAG ACATCTCTAA GCATTTGCTA CCTAG GA ACG TAC TGG TAC CAT AGC CAT Thr Tyr Trp Tyr His Ser His					
1053	1062	1071	1080	1089	1098
CTG GCC TTG CAG TAC TGT GAT GGG CTT CGC GGC CCC CTG GTG ATT TAC GAT CCC Leu Ala Leu Gln Tyr Cys Asp Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro					
1107	1116	1125	1134	1145	1155
CAT GAT CCG CAG GCA TAC CTG TAT GAC GTC GAT GAC G GTACCCAGCA CAGTTCCCT His Asp Pro Gln Ala Tyr Leu Tyr Asp Val Asp Asp Glu					

FIG.3B

1165	1175	1185	1198	1207	
AAAACCGTTA ACTTCTAATT CTGTAATAT CTTCATAG AG AGC ACC GTT ATC ACT CTG Ser Thr Val Ile Thr Leu					
1216	1225	1234	1243	1252	1267
GCA GAC TGG TAC CAT ACC CCG GCG CCT CTG CTG CCG CCT GCC GC GTACGCCCTCC Ala Asp Trp Tyr His Thr Pro Ala Pro Leu Leu Pro Pro Ala Ala					
1277	1287	1297	1307	1317	1328
ACACATCTGC ACAGCGTTCC GTATCTCATA CCCTAAACT TTATCGGACA G C ACT TTG ATT Thr Leu Ile					
1337	1346	1355	1364	1373	1382
AAT GGC CTG GGT CGC TGC CCT GGC AAC CCC ACC GCC GAC CTA GCC GTC ATC GAA Asp Gly Leu Gly Arg Trp Pro Gly Asn Pro Thr Ala Asp Leu Ala Val Ile Glu					
1391		1409	1419	1429	1439
GTC CAG CAC GGA AAG CG GTATGTCATA GCTCGGTTAT CTATTACATAC TCGCGGCCTC GAAGCTAAAA Val Gln His Gly Lys Arg					
1459	1470	1479	1488	1497	
CCTTGTTCGA G C TAC CGG TTC CGA CTG GTC AGC ACC TCA TGC GAC CCC AAC TAC Tyr Arg Phe Arg Leu Val Ser Thr Ser Cys Asp Pro Asn Tyr					
1506	1515	1524	1533	1542	1551
AAC TTC ACT ATC GAT GGC CAC ACC ATG ACA ATC ATC GAG GCG GAT GGG CAG AAC Asn Phe Thr Ile Asp Gly His Thr MET Thr Ile Ile Glu Ala Asp Gly Gln Asn					
1560	1569	1578	1587	1596	1605
ACC CAG CCA CAC CAA GTC GAC GGA CTT CAG ATC TTC GCG GCA CAG CGG TAC TCC Thr Gln Pro His Gln Val Asp Gly Leu Gln Ile Phe Ala Ala Gln Arg Tyr Ser					

FIG.3C

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1614	1627	1637	1647	1657	1667	
<u>TTC</u> <u>GTT</u> <u>GTATGTTTC</u> <u>CCGATTCGG</u> <u>AAAAAGGAAT</u> <u>TGGCGTGACA</u> <u>GCTCGAGTGT</u> <u>CCGTAG</u>						
Phe Val						
1676	1685	1694	1703	1712	1721	
<u>CTT</u> <u>AAC</u> <u>GCT</u> <u>AAC</u> <u>CAA</u> <u>GCG</u> <u>GTC</u> <u>AAC</u> <u>AAC</u> <u>TAC</u> <u>TGG</u> <u>ATC</u> <u>CGT</u> <u>GCG</u> <u>AAC</u> <u>CCT</u> <u>AAC</u> <u>CGT</u>						
Leu Asn Ala Asn Gln Ala Val Asn Asn Tyr Trp Ile Arg Ala Asn Pro Asn Arg						
1730	1739	1748	1757	1766	1775	
<u>GCT</u> <u>AAC</u> <u>ACT</u> <u>ACG</u> <u>GGC</u> <u>TTC</u> <u>GCC</u> <u>AAC</u> <u>GCC</u> <u>ATC</u> <u>AAC</u> <u>TCC</u> <u>GCC</u> <u>ATC</u> <u>CTG</u> <u>CGC</u> <u>TAC</u> <u>AAG</u>						
Ala Asn Thr Thr Gly Phe Ala Asn Gly Ile Asn Ser Ala Ile Leu Arg Tyr Lys						
1784	1793	1802	1811	1820	1829	
<u>GGG</u> <u>GCC</u> <u>CCG</u> <u>ATT</u> <u>AAG</u> <u>GAG</u> <u>CCT</u> <u>ACG</u> <u>ACG</u> <u>AAC</u> <u>CAG</u> <u>ACT</u> <u>ACC</u> <u>ATC</u> <u>CGG</u> <u>AAC</u> <u>TTT</u> <u>TTG</u>						
Gly Ala Pro Ile Lys Glu Pro Thr Thr Asn Gln Thr Thr Ile Arg Asn Phe Leu						
1838	1847	1856	1865	1874	1884	1894
<u>TGG</u> <u>GAG</u> <u>ACG</u> <u>GAC</u> <u>TTG</u> <u>CAC</u> <u>CCG</u> <u>CTC</u> <u>ACT</u> <u>GAC</u> <u>CCA</u> <u>CGT</u> <u>GCA</u> <u>GTAAAGTTCTA</u> <u>CACAGTCACC</u>						
Trp Glu Thr Asp Leu His Pro Leu Thr Asp Pro Arg Ala						
1904	1914	1924	1933	1942	1951	
<u>AACGGTGAGC</u> <u>TGTTGTCTGA</u> <u>TTGCACTGTG</u> <u>TTATAG</u> <u>CCT</u> <u>GCG</u> <u>CTT</u> <u>CCT</u> <u>TTC</u> <u>AAG</u> <u>GGG</u> <u>GCG</u>						
Pro Gly Leu Pro Phe Lys Gly Gly						
1960	1969	1978	1987	1997	2007	2017
<u>GTT</u> <u>GAC</u> <u>CAC</u> <u>GCT</u> <u>TTG</u> <u>AAC</u> <u>CTC</u> <u>AAC</u> <u>CTC</u> <u>ACT</u> <u>TTC</u> <u>GTACGTAGCG</u> <u>CCTCAGATAT</u> <u>CGACTAGTCT</u>						
Val Asp His Ala Leu Asn Leu Asn Leu Thr Phe						
2027	2037	2046	2055	2064	2073	
<u>ATCTCCTGAC</u> <u>CGATTGACAC</u> <u>AAT</u> <u>CCA</u> <u>TCG</u> <u>GAG</u> <u>TTC</u> <u>TTC</u> <u>ATC</u> <u>AAC</u> <u>GAT</u> <u>GCC</u> <u>CCT</u> <u>TTC</u>						
Asn Gly Ser Glu Phe Phe Ile Asn Asp Ala Pro Phe						

FIG.3D

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2082	2091	2100	2109	2118	2127
GTC CCT CCG ACT GTC CCG GTG CTA CTG CAG ATC CTG AAC GGA ACG CTC GAC GCG					
Val Pro Pro Thr Val Pro Val Ieu Leu Gln Ile Leu Asn Gly Thr Leu Asp Ala					
2136	2145	2154	2163	2172	2181
AAC GAC CTC CTG CCG CCC GGC AGC GTC TAC AAC CTT CCT CCG GAC TCC ACC ATC					
Asn Asp Leu Leu Pro Pro Gly Ser Val Tyr Asn Leu Pro Pro Asp Ser Thr Ile					
2190	2199	2208	2217	2226	2235
GAG CTG TCC ATT CCC GGA CGT GTG ACG GGT GGC CCG CAC CCA TTC CAT TTG CAC					
Glu Leu Ser Ile Pro Gly Gly Val Thr Gly Gly Pro His Pro Phe His Leu His					
2248	2258	2268	2278	2288	2297
GGG GTAATAATCT CTCTTTATAC TTTGGTCTCC CGATGCTGAC TTTCACTGCT CATCTTCAG					
Gly					
2306	2315	2324	2333	2342	2351
CAC GCT TTC TCC GTC GTG CGT AGC GCC GGC AGC ACC GAA TAC AAC TAC GCG AAC					
His Ala Phe Ser Val Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr Ala Asn					
2360	2369	2378	2387	2396	2405
CCG GTG AAG CGC GAC ACG GTC AGC ATT GGT CTT GCG GGC GAC AAC GTC ACC GTG					
Pro Val Lys Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp Asn Val Thr Val					
2414	2424	2434	2444	2454	2464
CGC TTC GTG GTATGTTTA CAGCCTCTCT ATCTCCGTGG GCGTTGGAA GTTGACTGGG CGCGTAG					
Arg Phe Val					
2474	2483	2492	2501	2510	2519
ACC GAC AAC CCC GGC CCG TGG TTC CTC CAC TGT CAC ATC GAC TTC CAT TTG CAA					
Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Gln					

FIG.3E

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2528	2537	2546	2555	2564	2573	
GCA GGC CTC GCC ATC GTG TTC GCG GAG GAC GCG CAG GAC ACG AAG CTT GTG AAC Ala Gly Leu Ala Ile Val Phe Ala Glu Asp Ala Gln Asp Thr Lys Leu Val Asn						
2582	2599	2609	2619	2629	2639	
CCC GTC CCT G GTACGTCTTC TGGATGCATG CGCTCCGCAC ACTGACTCAT CTTTGCAAC Pro Val Pro Glu						
2649	2658	2667	2676	2685		
AG AG GAC TGG AAC AAG CTG TGC CCC ACC TTC GAT AAG GCG ATG AAC ATC ACG Asp Trp Asn Lys Leu Cys Pro Thr Phe Asp Lys Ala MET Asn Ile Thr						
2694	2704	2714	2724	2734	2744	2754
GTT TCAGCGATGC GTGCCCTCA TGGTCATTT CTTGGAATCT TTGCATAGGG CTGCAGCACG Val						
2764	2774	2784	2794	2804	2814	2824
CTGGATACTC TTTCCCTTAG CAGGATATTA TTTAATGACC CCTCCGTTA GTGCTTAGTT AGCTTTACTA						
2834	2844	2854	2864	2874	2884	2894
CTGGTTGTAATGCTACCGCAGC ATGCCGTAATT CGGATAATGC TATCAATGTG TATATTATGA CACCGCTCAT						
2904	2914	2924	2934	2944	2954	2964
GCCCGATGCT TGAGTTGCAA GGTCCGTTTC CGATGCTCGA CATAAACGTT TCACTTACAT ACACATTGGG						
2974	2984	2994	3004	3014	3024	3034
TCTAGAACTG GATCTATCCA TGTATACAAA AACTCCTCAT ACAGCTGACT GGGGCCCTCT AGAGCATGGG						
3044	3054	3064	3074	3084	3094	3104
TCCGATTGAT CAGATGTCGC GAACACGAGC CTCCTGAGCT CGAGGACTCT GAGAAGCCCC CGTGCCTCT						

FIG.3F

10	20	30	40	50	60	70
GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG GAAAGCGGGC AGTGAGCCCA						
80	90	100	110	120	130	140
ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT						
150	160	170	180	190	200	210
GTTGTGTGGA ATTGTGAGCC GATAACAATT TCACACAGGA AACACCTATG ACATGATTAC GAATTCCGAT						
220	230	240	250	260	270	280
CGGCTTGGCC TCATTCCTCC ATGTTCCCCC GACCGAGCGG GCGCGTCAAT GGCCCGTTG CGAACACATA						
290	300	310	320	330	340	350
TGCAGGATAA ACAGTGCAGA ATATCAATGT GGCGGGGACA CAACCTCGCC GGCCGACACT CGACGCTGTT						
360	370	380	390	400	410	420
GATCATGATC ATGCTTGTG AGCATTCTAT ACGCAGCCTT GGAAATCTCA GGCGAATTG TCTGAATTGC						
430	440	450	460	470	480	490
GCTGGGAGGC TGGCAGCCCA GATCGGTGTG TCGGTGCAGT AGCCGACGCA GCACCTGGCC GAACCCGACA						
500	510	520	530	540	550	560
TCTCGGGTAC CACTTGATCT CCGCCAGATC ACTGCGGTTG CGCCATCGGC CGCGGGGCC ATTCTGTGTG						
570	580	590	600	610	620	630
TGCGCTGTAG CACTCTGCAT TCAGGCTCAA CGTATCCATG CTAGAGGACC GTCCAGCTGT TGGCCACGA						
640	650	660	670	680	690	700
TTCCGGCAGA AAGCTGTACA GGCAAGATATA AGGATGTCCC TCCGTCAGAG ACTCGTCACT CACAAGCCTC						

710 720 730 740 750 760 770
 TTTCCCTCTT CGCCTTCCA GCCTCTTCCA ACGCCTGCCA TCGTCCTCTT AGTCCTCTG TCCATTCTT
 780 790 799 808 817 826
 CTGCGTAGTT AATC ATG GGC AGG TTC TCA TCT CTC TGC GCG CTC ACC GCC GTC ATC
 MET Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile
 835 844 853 862 871 880
 CAC TCT TTT GGT CGT GTC TCC GCC GCT ATC GGG CCT GTG ACC GAC CTC ACC ATC
 His Ser Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile
 889 898 907 916 925 934
 TCC AAT GGG GAC GTT TCT CCC GAC GGC TTC ACT CGT GCC GCA GTG CTT GCA AAC
 Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu Ala Asn
 943 952 961 970 980 990
 GGC GTC TTC CCG GGT CCT CTT ATC ACG GGA AAC AAG GTACGTGGCA TGGCTTCAGT
 Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys
 1000 1010 1020 1029 1038 1047
 CTACACCTA CAAGCCTTCT AACTCTTTA CCACAG GGC GAC AAC TTC CAG ATC AAT GTT
 Gly Asp Asn Phe Gln Ile Asn Val
 1056 1065 1074 1083 1092 1105
 ATC GAC AAC CTC TCT AAC GAG ACG ATG TTG AAG TCG ACC TCC ATC GTATGTGCTT
 Ile Asp Asn Leu Ser Asn Glu Thr MET Leu Lys Ser Thr Ser Ile
 1115 1125 1135 1145 1156 1165
 CTACTGCTTC TTAGTCTTGG CAATGGCTCA AGCTCTCCTC CGCAG CAT TGG CAC GGC TTC
 His Trp His Gly Phe

FIG. 4B

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1174	1183	1192	1201	1210	1219
TTC CAG AAG GGT ACT AAC TGG GCT GAT GGA GCT GCC TTC GTC AAC CAG TGC CCT Phe Gln Lys gly thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro					
1228	1237	1246	1235	1264	
ATC GCG ACG GGG AAC TCT TTC CTT TAC GAC TTC ACC GCG ACG GAC CAA GCA G Ile Ala Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala Gly					
1281	1291	1301	1311	1321	1331
GTCAGTGCCT GTGGCCCTTA TCTTTCCCG TAATCAGCAG CTAACACTCC GCACCCACAG GC					
1342	1351	1360	1369	1378	1387
ACC TTC TGG TAC CAC AGT CAC TTG TCT ACG CAG TAC TGC GAT GGT TTG CGG GGC Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly					
1396	1405	1414	1423	1432	1441
CCG ATG GTC GTC TAC GAC CCG AGT GAC CCG CAT GCG GAC CTT TAC GAC GTC GAC Pro MET Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp Leu Tyr Asp Val Asp					
1450	1459	1468	1477	1486	1495
GAC GAG ACC ACG ATC ATC ACG CTC TCT GAT TGG TAT CAC ACC GCT GCT TCG CTC Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp Tyr His Thr Ala Ala Ser Leu					
1504	1519	1529	1539	1549	1559
GGT GCT GCC TTC CC GAACTTTAC CCCAGCCAC GGAGTTAAGA CCGATCTAA CTGTAATACG Gly Ala Ala Phe Pro					
1568	1577	1586	1604	1614	
TTCAG G ATT GGC TCG GAC TCT ACC CTG ATT AAC GG GTTGGCCGCT TCGCGGGTGG Ile Gly Ser Asp Ser Thr Leu Ile Asn Gly					

FIG.4C

1624	1633	1642	1651	1669	
TGACAG C ACT GAC CTT GCG GTT ATC ACT GTC GAG CAG GGC AAG CG GTTAGTGATA					
Thr Asp Leu Ala Val Ile Thr Val Glu Gin Gly Lys Arg					
1679	1689	1699	1709	1719	1728
CCCTCTACAG TTGACACTGT GCCATTGCTG ACAGTACTCT CAG C TAC CGT ATG CGT CTT					
Tyr Arg MET Arg Leu					
1737	1746	1755	1764	1773	1782
CTC TCG CTG TCT TGC GAC CCC AAC TAT GTC TTC TCC ATT GAC GGC CAC AAC ATG					
Leu Ser Leu Ser Cys Asp Pro Asn Tyr Val Phe Ser Ile Asp Gly His Asn MET					
1791	1800	1809	1818	1827	1836
ACC ATC ATC GAG GCC GAC GCC GTC AAC CAC GAG CCC CTC ACG GTT GAC TCC ATC					
Thr Ile Ile Gin Ala Asp Ala Val Asn His Glu Pro Leu Thr Val Asp Ser Ile					
1845	1854	1863	1879	1889	1899
CAG ATC TAC GCC GGC CAA CGT TAC TCC TTC GTC GTACGTATT CGAACAGCCA TGATCACGCC					
Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Phe Val					
1909	1919	1928	1937	1946	1955
AAGCCCGATC CTAACGGGCC TACCCCTAG CTT ACC GCT GAC CAG GAC ATC GAC AAC TAC					
Leu Thr Ala Asp Gln Asp Ile Asp Asn Tyr					
1964	1973	1982	1991	2000	2009
TTC ATC CGT GCC CTG CCC AGC GCC GGT ACC ACC TCG TTC GAC GGC GGC ATC AAC					
Phe Ile Arg Ala Leu Pro Ser Ala Gly Thr Thr Ser Phe Asp Gly Ile Asn					
2018	2027	2036	2045	2054	2063
TCG GCT ATC CTG CGC TAC TCT GGT GCC TCC GAG GTT GAC CCG ACC ACG GAG					
Ser Ala Ile Leu Arg Tyr Ser Gly Ala Ser Glu Val Asp Pro Thr Thr Glu					

FIG.4D

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2072	2081	2090	2099	2108	2117												
ACC	ACC	AGC	GTC	CTC	CCC	CTC	GAC	GAG	GCG	AAC	CTC	GTG	CCC	CTT	GAC	AGC	CCC
Thr	Thr	Ser	Val	Leu	Pro	Leu	Asp	Glu	Ala	Asn	Leu	Val	Pro	Leu	Asp	Ser	Pro
2126	2136	2146	2156	2166	2176												
GCT	GCT	GTACGTCGTA	TTCTGCCCTT	GCAAGGATCG	CACATACTAA	CATGCTCTTG	TAG	CCC									
Ala	Ala																Pro
2185	2194	2203	2212	2221	2230												
GGT	GAC	CCC	AAC	ATT	GGC	GGT	GTC	GAC	TAC	GCG	CTG	AAC	TTG	GAC	TTC	AAC	TTC
Gly	Asp	Pro	Asn	Ile	Gly	Gly	Val	Asp	Tyr	Ala	Leu	Asn	Leu	Asp	Phe	Asn	Phe
2239	2248	2257	2266	2275	2284												
GAT	GGC	ACC	AAC	TTC	TTC	ATC	AAC	GAC	GTC	TCC	TTC	GTG	TCC	CCC	ACG	GTC	CCT
Asp	Gly	Thr	Asn	Phe	Phe	Ile	Asn	Asp	Val	Ser	Phe	Val	Ser	Pro	Thr	Val	Pro
2293	2302	2311	2320	2329	2338												
GTC	CTC	CTC	CAG	ATT	CTT	AGC	GGC	ACC	ACC	TCC	GCG	GCC	GAC	CTT	CTC	CCC	AGC
Val	Leu	Leu	Gln	Ile	Leu	Ser	Gly	Thr	Thr	Ser	Ala	Ala	Asp	Leu	Leu	Pro	Ser
2347	2356	2365	2374	2383	2392												
GGT	AGT	CTC	TTC	GGC	GGC	CCG	CCG	TCC	AAC	TCG	ACG	ATC	GAG	ATC	TCG	TTC	CCC
Gly	Ser	Leu	Phe	Ala	Val	Pro	Ser	Asn	Ser	Thr	Ile	Glu	Ile	Ser	Phe	Pro	Ile
2401	2410	2419	2428	2437	2446	2456											
ACC	GCG	ACG	AAC	GCT	CCC	GGC	GGC	CCG	CAT	CCC	TTC	CAC	TTG	CAC	GGT	GTACGTCGTC	
Thr	Ala	Thr	Asn	Ala	Pro	Gly	Ala	Pro	His	Pro	Phe	His	Leu	His	Gly		
2466	2476	2486	2496	2506	2515												
CATCTCATAT	GCTACGGAGC	TCCACGCTGA	CCGCCCTATA	G	CAC	ACC	TTC	TCT	ATC	GTT							

FIG.4E

2524	2533	2542	2551	2560	2569	
CGT ACC GCC GGC AGC ACG GAT ACG AAC TTC GTC AAC CCC GTC CGC CGC GAC GTC						
Arg Thr Ala Gly Ser Thr Asp Thr Asn Phe Val Asn Pro Val Arg Arg Asp Val						
2578	2587	2596	2605	2614	2624	
GTG AAC ACC GGT ACC GTC GGC GAC AAC GTC ACC ATC CGC TTC ACC GTACGGCAGCA						
Val Asn Thr Gly Thr Val Gly Asp Asn Val Thr Ile Arg Phe Thr						
2634	2644	2654	2664	2673	2682	
CTCTCCTAAC ATTCCCCACTG CGCGATCACT GACTCCTCGC CCACAG ACT GAC AAC CCC GGC						
Thr Asp Asn Pro Gly						
2691	2700	2709	2718	2727	2736	
CCC TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC TTG GAG GCC GGT TTC GCC ATC						
Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile						
2745	2754	2763	2772	2781		2798
GTC TTC AGC GAG GAC ACC GCC GAC GTC TCG AAC ACG ACC ACG CCC TCG A GTACGTTGTC						
Val Phe Ser Glu Asp Thr Ala Asp Val Ser Asn Thr Thr Pro Ser Thr						
2808	2818	2828	2838	2850	2859	
CTCCCGTGCC CATCTCCGGC CGCCTGACTA ACCGAGCACCC CTTACAG CT GCT TGG GAA GAT						
Ala Trp Glu Asp						
2868	2877	2886	2895	2908	2918	
CTG TGC CCC ACG TAC AAC GCT CTT GAC TCA TCC GAC CTC TAATCGGTTCAAAAGGTGCGC						
Leu Cys Pro Thr Tyr Asn Ala Leu Asp Ser Ser Asp Leu						
2928	2938	2948	2958	2968	2978	2988
TCGCTACCTT AGTAGGTAGA CTTATGCACC GGACATTATC TACAATGGAC TTTAATTGCGCTAACGGCC						
2998	3008	3018	3028	2038	3048	3058
GTATACATA CGCCGACGTA GTATAAGGT TCTCTGGATT GGTCGGACCT ACAGACTGCA ATTTCGTGA						
3068	3078	3088	3098			
CCTATCAACT GTATATTGAA GCACGGAGT GAATGGAAAT AGAGACA						

10	20	30	40	50	60	70
CTCATAACTC TTGGCTTCTA GCATGGGGC TGGGCACACC TGACAGACCC TTGGGAGGC GAACTCGAAT						
80	90	100	110	120	130	140
GCAGCGTACT CTATCNCACC TCCAGGAAAG GTAGGGATGG ACNCCGTGCA CCAACAACTG TCTCTCCACC						
150	160	170	180	190	200	210
AGCAACCATC CCTGGATAT GTCTCCACAC ACCCGGTGTC TACAAGCGG GATCTGTGCT GGTGAAGTGC						
220	230	240	250	260	270	280
TGTCTCEGGA CGGGGGCGG CGAGCGACCA GAACCCGAAC CAGTGTAGT GCGGACACC CGCGAGACAA						
290	300	310	320	330	340	350
<u>TTGTGCAGGG TGAGTTATAT TCTTGTGAG ACGGGCTGC CGCTCGGCAC TGAAAGCGTC GCAGTTAGGT</u>						
360	370	380	390	400	410	420
GATCCAGCGG TCCGGCTAT TTTGACGTC TGGCAGCTAT CCTAAGCCGC GCCTCCATAC ACCCCAGGG						
430	440	450	460	470	480	490
CTCTCGTTG CTATAGGTAT AAATCCCTCA GCTTCAGAGC GTCGATCCTC ATCCCACACG ACACCCGTT						
500	510	520	530	540	550	
CAGTCTTCTC GTAGCCATT CCCTAGCCGC CCAGCCTCCG CTTTGTGTTT CAAC ATG GGC AAG MET Gly Lys						
559	568	577	586	595	604	
TAT CAC TCT TTT GTG AAC GTC GTC GCC CTT AGT CTT TCT TTG AGC GGT CCT GTG Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu Ser Leu Ser Gly Arg Val						
613	622	631	640	649	658	
TTC GGC GCC ATT GGG CCC GTC ACC GAC TTG ACT ATC TCT AAC GCC GAT GTT ACG Phe Gly Ala Ile Gly Pro Val Thr Asp Leu Thr Ile Ser Asn Ala Asp Val Thr						

FIG.5A

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667	676	685	694	703	712
CCT GAC GGC ATT ACT CGT GCT GCT GTC CTC GCG GGC GGC GTT TTC CCC GGG CCC Pro Asp Gly Ile Thr Arg Ala Ala Val Leu Ala Gly Gly Val Phe Pro Gly Pro					
721	730	743	753	763	773
CTC ATT ACC GGC AAC AAG GTGAGCCGG AAACCTTCTA CTAGCCGGCT CGTACCGGTGC ACCGTTACTG Leu Ile Thr Gly Asn Lys					
793	803	814	823	832	841
AAGCCACACT TTGCGCTGTC AACAG GGG GAT GAA TTC CAG ATC AAT GTC ATC GAC AAC Gly Asp Glu Phe Gln Ile Asn Val Ile Asp Asn					
850	859	868	877	887	897
CTG ACC AAC GAG ACC ATG TTG AAG TCG ACC ACA ATC GTAAAGGTGCT TGCTCCCATA Leu Thr Asn Glu Thr MET Leu Lys Ser Thr Thr Ile					
907	917	927	938	947	956
ATTAAGCCCG TCGCTGACTC GAAAGTTATC TGTAG CAC TGG CAT GGT ATC TTC CAG GCC His Trp His Gly Ile Phe Gln Ala					
965	974	983	992	1001	1010
GGC ACC AAC TCG GCA GAC GGC GCG GCC TTC GTG AAC CAG TGC CCT ATC GCC ACG Gly Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr					
1019	1028	1037	1046		1063
GGA AAC TCG TTC TTG TAC GAC TTC ACC GTT CCT GAT CAA GCC G GTACGTTAT Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp Gln Ala Gly					
1073	1083	1093	1103	1112	1121
ACACTTCCCT TTCTCCGGCA TACTCTGACG CGCCGCTGGA TCAG GC ACC TTC TGG TAC CAC Thr Phe Trp Tyr His					

FIG.5B

1130	1139	1148	1157	1166	1175
AGC CAC CTG TCC ACC CAG TAC TGT GAC GGC CTG CGC GGT CCT CTT GTG GTC TAC Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Leu Val Val Tyr					
1184	1193	1202	1211	1220	1231
GAC CCC GAC GAT CCC AAC GCG TCT CTT TAC GAC GTC GAT GAC G GTAAGCAGGC Asp Pro Asp Asp Pro Asn Ala Ser Leu Tyr Asp Val Asp Asp Asp					
1241	1251	1261	1271	1281	1290
TACTTGTGGA CTTGTATGGA TGTATCTCAC GCTCCCCTAC AG AT ACT ACG GTT ATT ACG Thr Thr Val Ile Thr					
1299	1308	1317	1326	1335	1347
CTT GCG GAC TGG TAC CAC ACT GCG GCG AAG CTG GGC CCT GCC TTC CC GTGAGTCTAC Leu Ala Asp Trp Tyr His Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro					
1357	1367	1377	1387	1397	1408
TCTTCCTCGT GTGTTAACAT AGGTGACGGC CGCTGATACG AGAGCTACCA G C GCG GGT CCG Ala Gly Pro					
1417	1426	1435	1444	1453	1462
GAT AGC GTC TTG ATC AAT GGT CTT GGT CGG TTC TCC GGC GAT GGT GGA GGA GCG Asp Ser Val Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly Gly Ala					
1471	1480	1489	1498	1510	1520
ACA AAC CTC ACC GTG ATC ACC GTC ACG CAA GGC AAA CG GTGAGTCCGC CCTGAGCTGG Thr Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg					
1530	1540	1550	1561	1570	1579
CCTCAATAGC GATATTGACG AGTCCATGCC CTCCAG G TAC CGC TTC CGC CTT GTG TCG Tyr Arg Phe Arg Leu Val Ser					

FIG.5C

1588	1597	1606	1615	1624	1633
ATC TCG TCC GAC CCC AAC TTC ACC TTC TCG ATC GAC GGG CAC AAC ATG ACC ATC	Ile Ser Cys Asp Pro Asn Phe Thr Phe Ser Ile Asp Gly His Asn MET Thr Ile				
1642	1651	1660	1669	1678	1687
ATC GAG GTG GAC GGT GTC AAC CAC GAG GCC TTG GAC GTC GAC TCC ATT CAG ATT	Ile Glu Val Asp Gly Val Asn His Glu Ala Leu Asp Val Asp Ser Ile Gln Ile				
1696	1705	1714	1724	1734	1744
TTT GCG GGG CAG CGG TAC TCC TTC ATC GTACGTTCCC TTGCCCTCGT CCTATATCCG	Phe Ala Gly Gln Arg Tyr Ser Phe Ile				
1754	1764	1774	1785	1794	1803
CCCCGTCTGCT CACAGAGGCT TCTATATCCG AG CTC AAC GCC AAC CAG TCC ATC GAC AAC	Leu Asn Ala Asn Gln Ser Ile Asp Asn				
1812	1821	1830	1839	1848	1857
TAC TGG ATC CGC GCG ATC CCC AAC ACC GGT ACC ACC GAC ACC ACG GGC GGC GTC	Tyr Trp Ile Arg Ala Ile Pro Asn Thr Gly Thr Thr Asp Thr Thr Gly Gly Val				
1866	1875	1884	1893	1902	1911
AAC TCT GCT ATT CTT CGC TAC GAC ACC GCA GAA GAT ATC GAG CCT ACG ACC AAC	Asn Ser Ala Ile Leu Arg Tyr Asp Thr Ala Glu Asp Ile Glu Pro Thr Thr Asn				
1920	1929	1938	1947	1956	1965
GCG ACC ACC TCC GTC ATC CCT CTC ACC GAG ACG GAT CTG GTG CCG CTC GAC AAC	Ala Thr Thr Ser Val Ile Pro Leu Thr Glu Thr Asp Leu Val Pro Leu Asp Asn				
1974	1983	1992	2001	2010	2019
CCT GCG GCT CCC GGT GAC CCC CAG GTC GGC GGT GTT GAC CTG GCT ATG AGT CTC	Pro Ala Ala Pro Gly Asp Pro Gln Val Gly Gly Val Asp Leu Ala MET Ser Leu				

2028	2041	2051	2061	2071	2081
<u>GAC</u> <u>TTC</u> <u>TCC</u> <u>TTC</u> <u>CTGAGTCCCA</u> CAGGACTCCG CGCCATTCC CTTATTTACG CAGGAGTATT Asp Phe Ser Phe					
2090	2099	2108	2117	2126	2135
<u>GTTCAG</u> <u>AAC</u> <u>GGT</u> <u>TCC</u> <u>AAC</u> <u>TTC</u> <u>TTT</u> <u>ATC</u> <u>AAC</u> <u>AAC</u> <u>GAG</u> <u>ACC</u> <u>TTC</u> <u>GTC</u> <u>CCG</u> <u>CCC</u> <u>ACA</u> Asn Gly Ser Asn Phe Phe Ile Asn Asn Glu Thr Phe Val Pro Pro Thr					
2144	2153	2162	2171	2180	2189
<u>GTT</u> <u>CCC</u> <u>GTG</u> <u>CTC</u> <u>CTG</u> <u>CAG</u> <u>ATT</u> <u>TTG</u> <u>AGT</u> <u>GGT</u> <u>GCG</u> <u>CAG</u> <u>GAC</u> <u>GCG</u> <u>GCG</u> <u>AGC</u> <u>CTG</u> <u>CTC</u> Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Gln Asp Ala Ala Ser Leu Leu					
2198	2207	2216	2225	2234	2243
<u>CCC</u> <u>AAC</u> <u>GGG</u> <u>AGT</u> <u>GTC</u> <u>TAC</u> <u>ACA</u> <u>CTC</u> <u>CCT</u> <u>TCG</u> <u>AAC</u> <u>TCG</u> <u>ACC</u> <u>ATT</u> <u>GAG</u> <u>ATC</u> <u>TCG</u> <u>TTC</u> Pro Asn Gly Ser Val Tyr Thr Leu Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe					
2252	2261	2270	2279	2288	2297
<u>CCC</u> <u>ATC</u> <u>ATC</u> <u>ACC</u> <u>ACC</u> <u>GAC</u> <u>GGT</u> <u>GTT</u> <u>CTG</u> <u>AAC</u> <u>GCG</u> <u>CCC</u> <u>GGT</u> <u>GCT</u> <u>CCG</u> <u>CAC</u> <u>CCG</u> <u>TTC</u> Pro Ile Ile Thr Thr Asp Gly Val Leu Asn Ala Pro Gly Ala Pro His Pro Phe					
2306	2319	2329	2339	2349	2359
<u>CAT</u> <u>CTC</u> <u>CAC</u> <u>GCC</u> <u>GTAAAGTCCTT</u> <u>GCTTTCCTCA</u> <u>GTGCCTCGCT</u> <u>TCCACGACGT</u> <u>CCACTGATCC</u> His Leu His Gly					
2369	2380	2389	2398	2407	2416
<u>CACACATCCC</u> <u>ATGTGCAG</u> <u>CAC</u> <u>ACC</u> <u>TTC</u> <u>TCG</u> <u>GTG</u> <u>GTG</u> <u>CGC</u> <u>AGC</u> <u>GCC</u> <u>GGG</u> <u>AGC</u> <u>TCG</u> <u>ACC</u> His Thr Phe Ser Val Val Arg Ser Ala Gly Ser Ser Thr					
2425	2434	2443	2452	2461	2470
<u>TTC</u> <u>AAC</u> <u>TAC</u> <u>GCC</u> <u>AAC</u> <u>CCA</u> <u>GTC</u> <u>CGC</u> <u>CGG</u> <u>GAC</u> <u>ACC</u> <u>GTC</u> <u>AGT</u> <u>ACT</u> <u>GGT</u> <u>AAC</u> <u>TCT</u> <u>GGC</u> Phe Asn Tyr Ala Asn Pro Val Arg Arg Asp Thr Val Ser Thr Gly Asn Ser Gly					

2479	2488	2504	2514	2524	2534
<pre> GAC AAC GTC ACT ATC CGC TTC ACG GTACGTCCTC TCCGGAGCCC TCCCACCCGT GTGTCCGCTG Asp Asn Val Thr Ile Arg Phe Thr </pre>					
2544	2554	2564	2574	2583	2592
<pre> ACCGCTAAC ACCGCCCCACC GTGCTGCTGC TGCGCAG ACC GAC AAC CCA GGC CCG TGG Thr Asp Asn Pro Gly Pro Trp </pre>					
2601	2610	2619	2628	2637	2646
<pre> TTC CTC CAC TGC CAC ATC GAC TTC CAC CTG GAG GCC GGC TTC GCC ATC GTC TGG Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Trp </pre>					
2655	2664	2673	2682	2699	
<pre> GGG GAG GAC ACT GCG GAC ACC GCG TCC GCC AAT CCC GTT CCT A GTACGTCGTG Gly Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro Thr </pre>					
2709	2710	2729	2739	2749	2759
<pre> CCTGCTGAGC TCTTGTGCC CCAACAGGGT CCTGATCGTC CCTTCCTCCG TGCAG CG GCG TGG Ala Trp </pre>					
2768	2777	2786	2795	2804	2817
<pre> > AGC GAT TTG TGC CCC ACT TAC GAT GCT TTG GAC TCC TCC GAC CTC TGATCGACAA Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser Ser Asp Leu </pre>					
2827	2837	2847	2857	2867	2877
<pre> GGCATGAAGG CTGAAGCAGC TGCGGTCAAT TCTCGAACAC ACTTTACTCG AACATTCAATT TTCTTTGGC </pre>					
2897	2907	2917			
<pre> TCGGGATCGG AACAAATCAT GGGGGGGCCG GACCGTCT </pre>					

FIG.5F

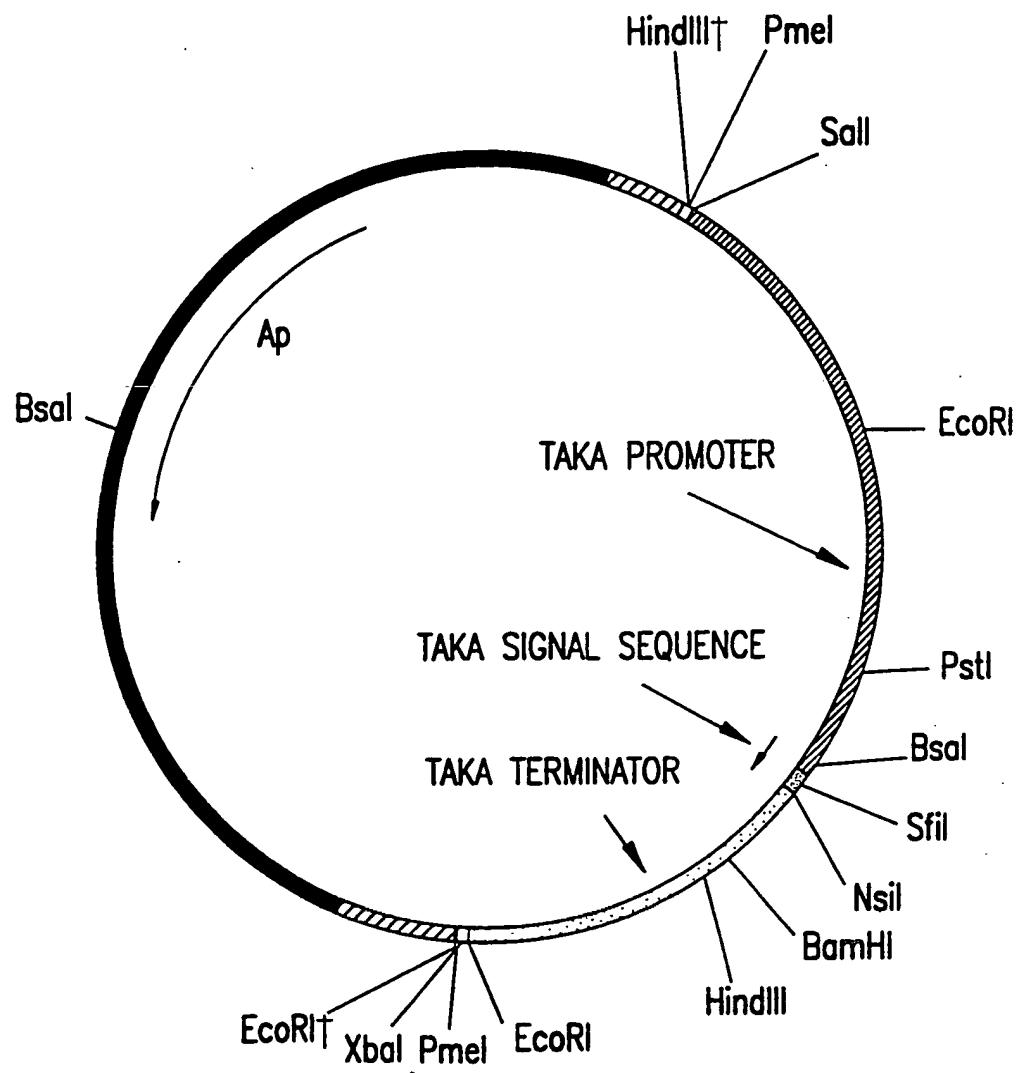


FIG.6

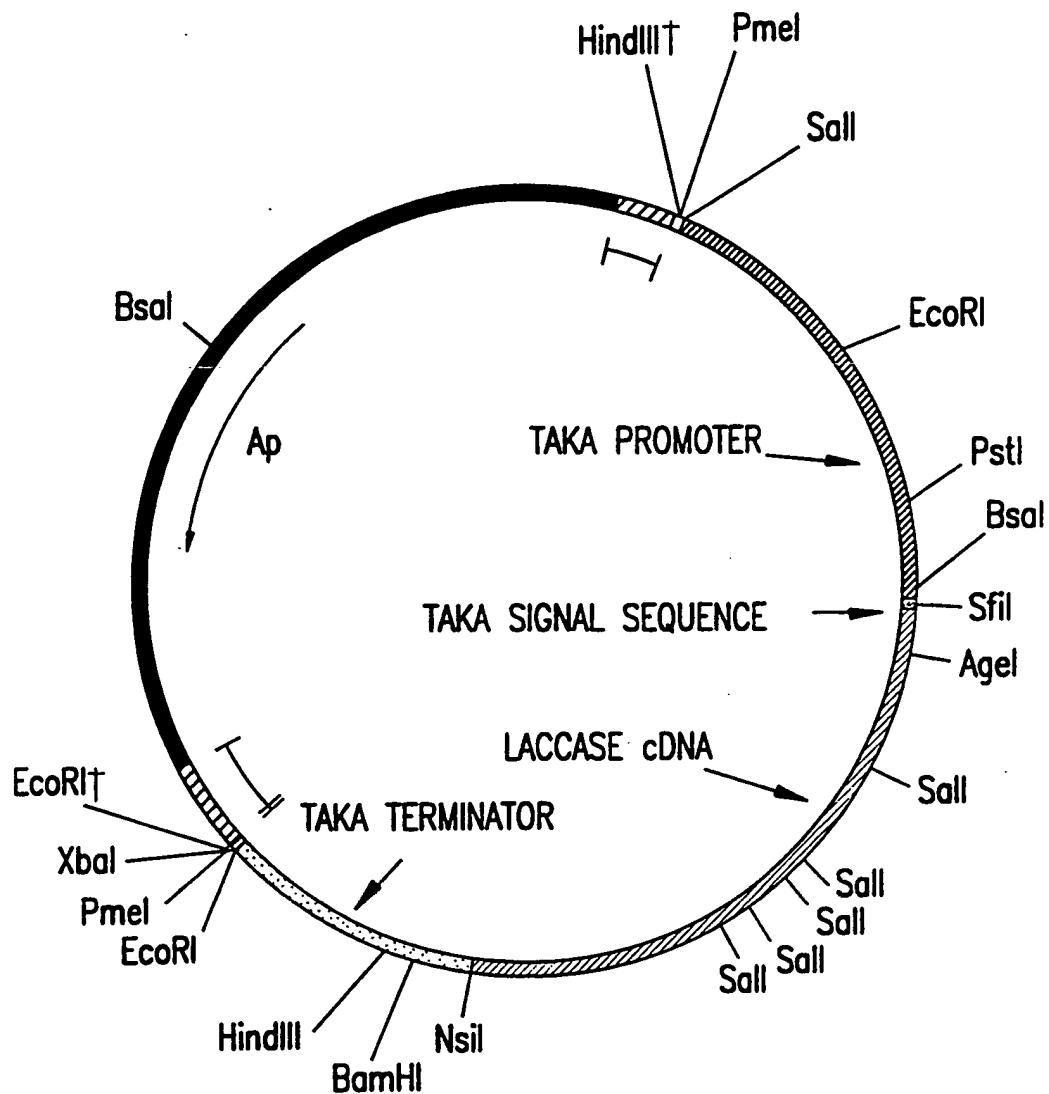


FIG.7

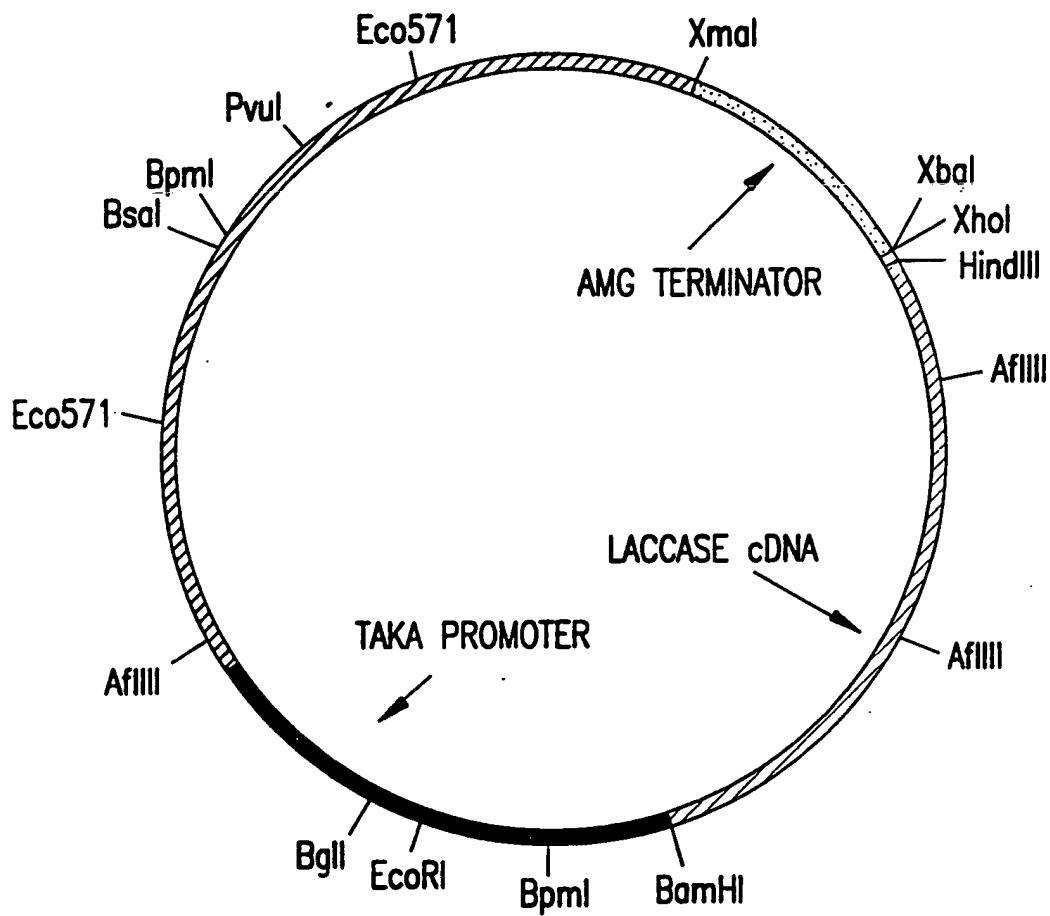


FIG.8

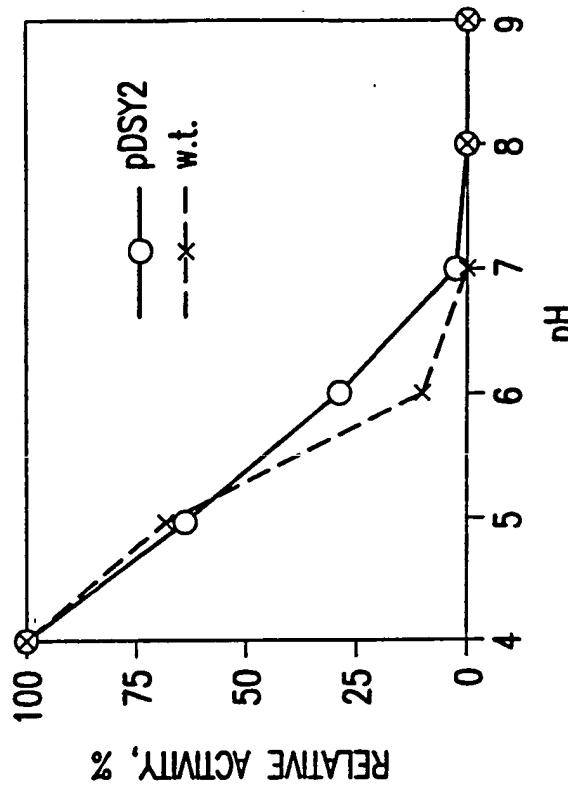


FIG. 9B

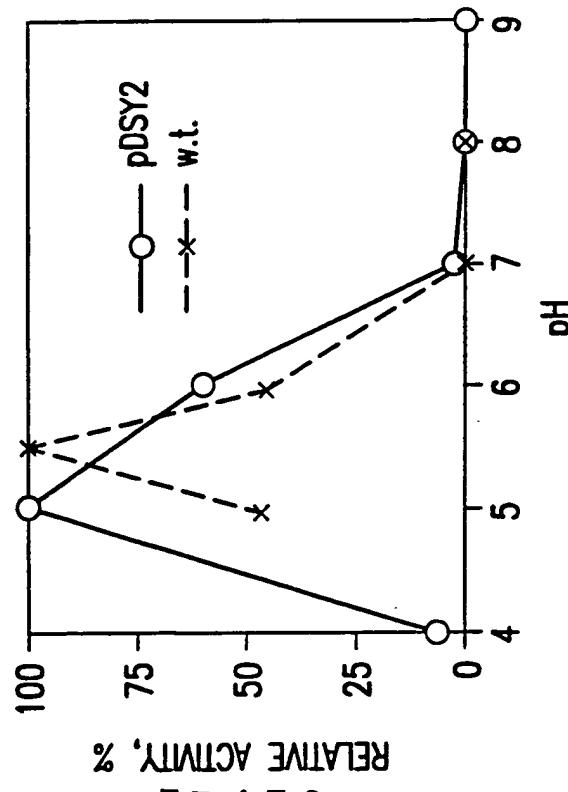


FIG. 9A

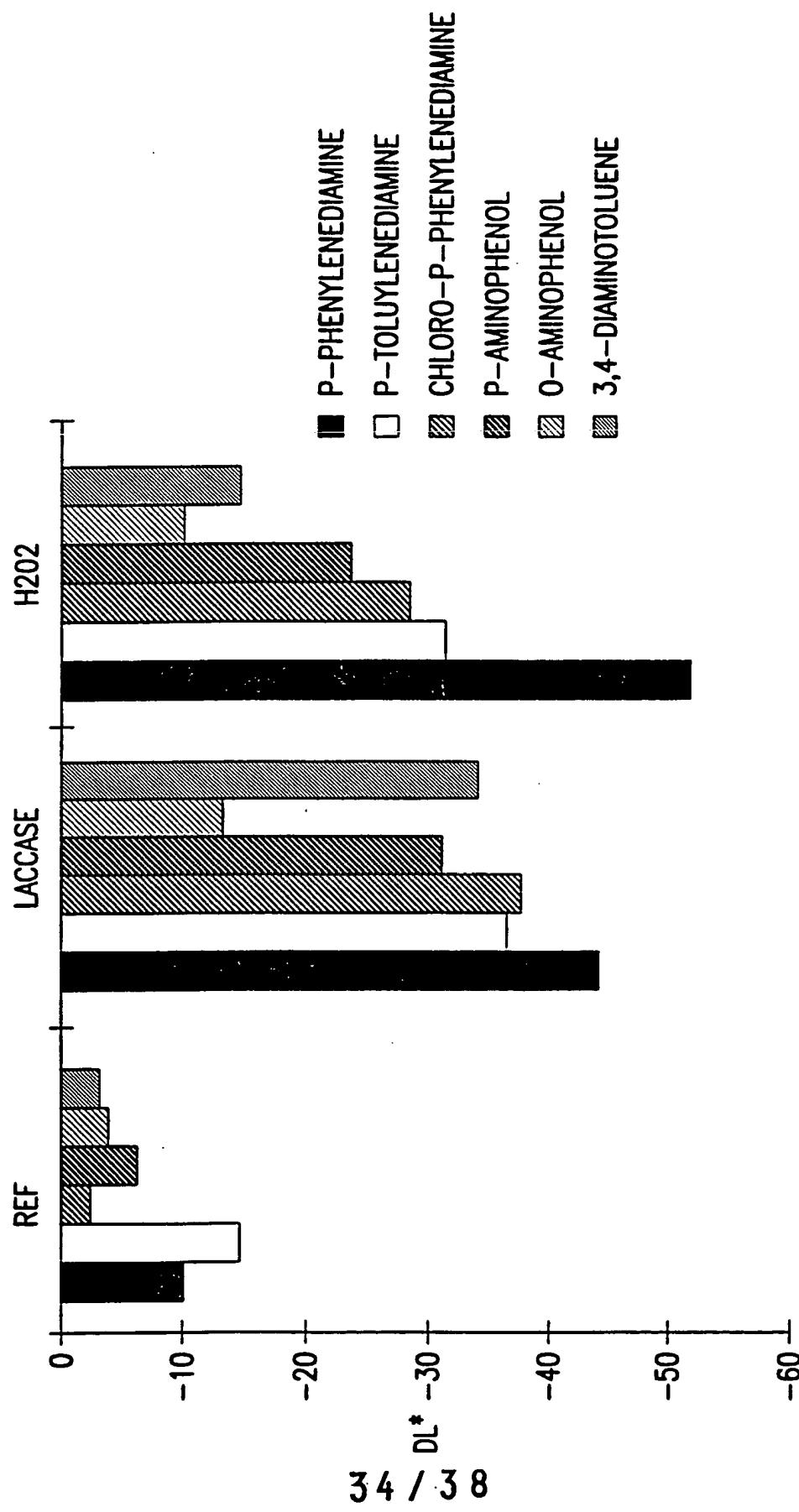


FIG. 10

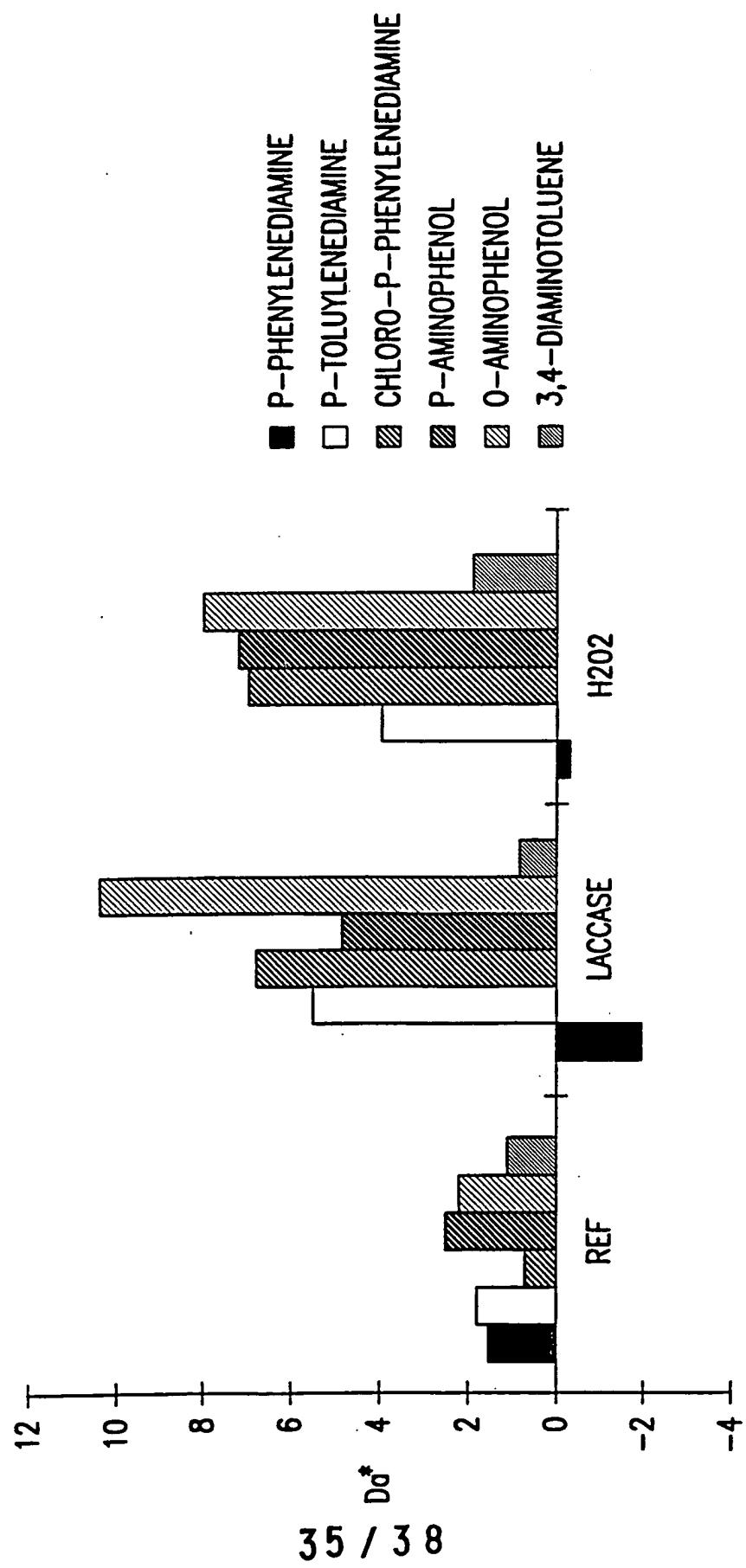


FIG. 11

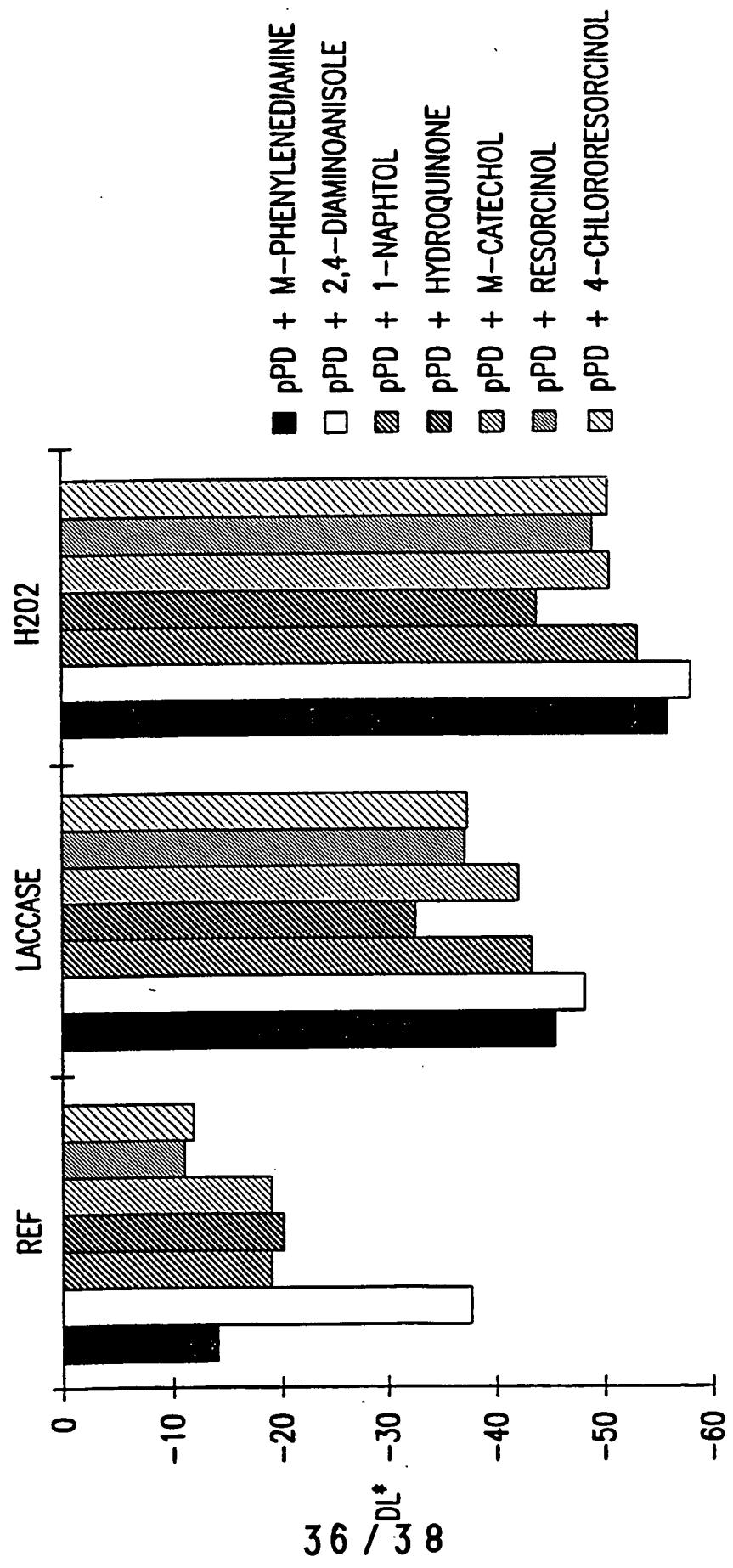


FIG.12

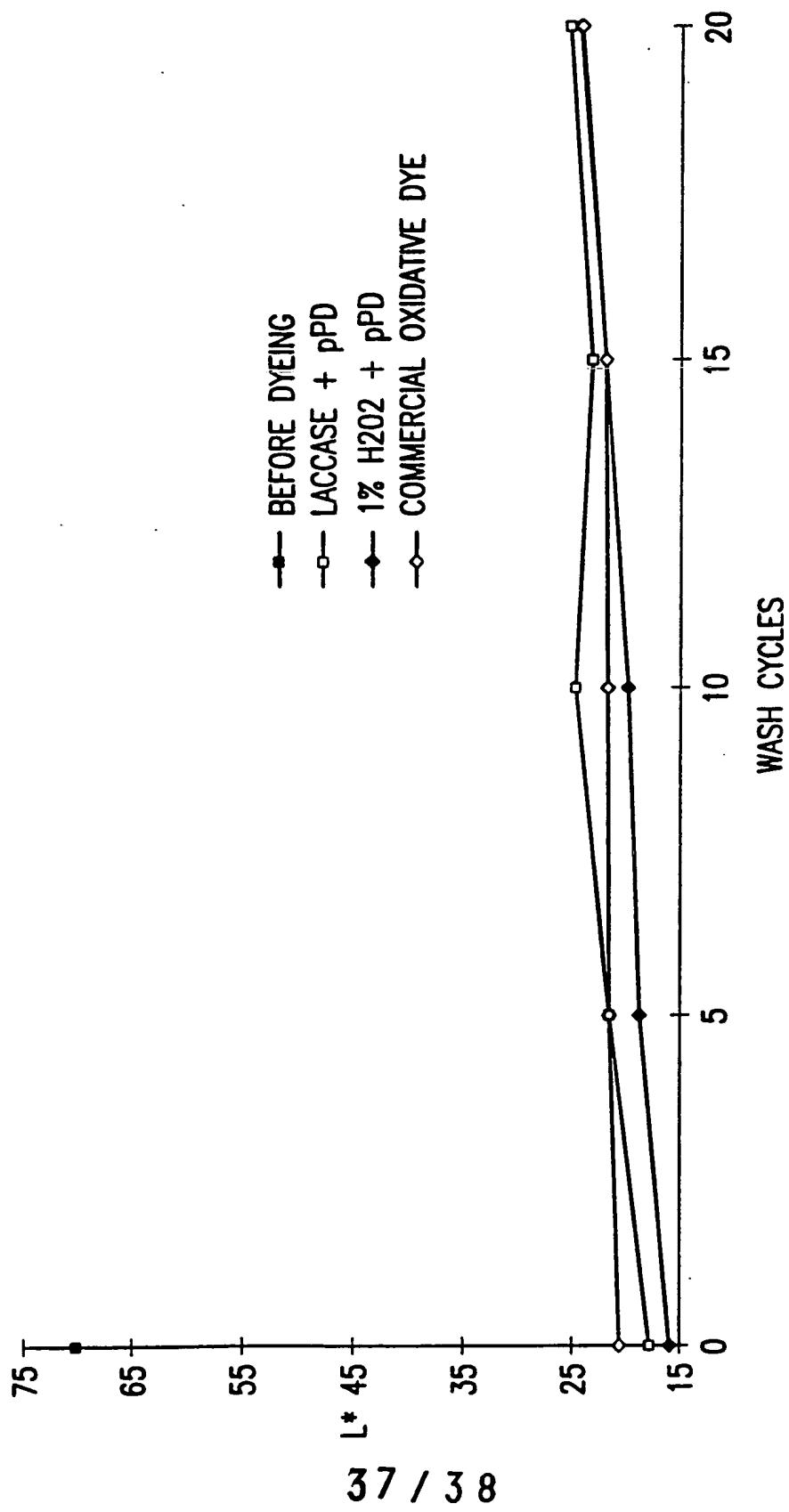


FIG. 13

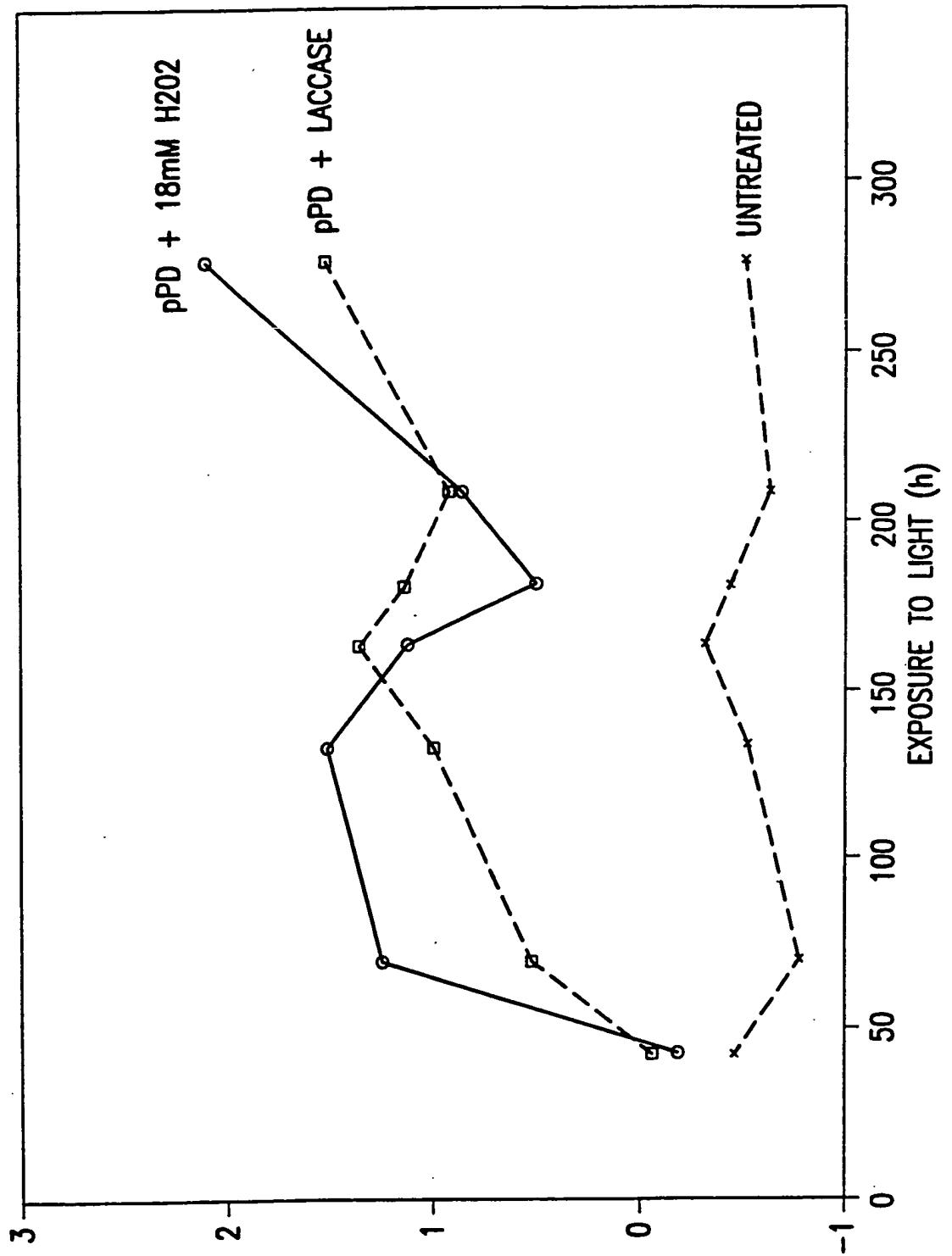


FIG. 14

A. CLASSIFICATION OF SUBJECT MATTER			C12N1/15	A61K7/13	A61K7/06
IPC 6	C12N15/53	C12N9/02			
	D21C5/00	C12N15/80	//(C12N1/15,C12R1:66)		

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	GEN. TECH. REP. NC (NORTH CENT. FOR EXP. STN.), vol. 175, 1994 pages 115-118, YAVER D.S. ET AL. 'The molecular cloning and expression of laccase genes from the white-rot basidiomycete <i>Polyporus pinsitu</i> ' see the whole document ---	1-48
P, X	WO,A,95 01426 (NOVONORDISK AS ;SCHNEIDER PALLE (DK); PEDERSEN ANDERS HJELHOLT (DK) 12 January 1995 see page 6 - page 7; claim 22; example 2 ---	15-17, 35-41, 45, 48
X	DE,C,40 33 246 (PFLEIDERER UNTERNEMENSVERWALTUNG GMBH & CO.) 27 February 1992 see the whole document ---	15, 16, 35

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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